THE EFFECTS OF PROGESTERONE ON INFLUENZA PATHOGENESIS IN FEMALE C57BL/6 MICE

by

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ABSTRACT

Over 100 million women worldwide are currently on progestin-based hormonal contraceptives. Despite having immunomodulatory and repair properties, their effects on the outcome of viral diseases outside of the reproductive tract have not been evaluated. I hypothesized that treatment of influenza A virus-infected female mice with progesterone or a synthetic analog, levonorgestrel, would protect them against severe disease. Female mice treated with progestins were significantly protected against infection with different strains and doses of influenza A virus as compared to females treated with placebo. This protection was not caused by a reduction in virus titers, but rather by decreased pulmonary inflammation and tissue damage, increased proliferation, and improved pulmonary function during infection. Treatment with progesterone reduced inflammation and promoted repair by inducing TGF-β, IL-22, and suppressive CD39+ Th17 cells along with the production of amphiregulin. Amphiregulin mediated progesterone-induced repair as treatment of progesterone-depleted mice with amphiregulin restored protection whereas depletion of amphiregulin in progesterone-treated females impaired protection during IAV infection. *In vitro*, progesterone facilitated the repair of damaged respiratory epithelial cell cultures and induced amphiregulin production. To investigate whether adaptive immune responses and the outcome of sequential influenza A virus infection were affected by progestins, mice were treated with progestins, infected with H1N1 virus, and challenged with either a drift variant or a H3N2 virus. Treatment with progestins reduced virus-specific antibody titers in

the serum and bronchoalveolar lavage fluid. Protection following infection with the drift variant virus was similar among all groups and correlated with high titers of broadly neutralizing antibodies. In contrast, mice treated with progestins suffered greater mortality following a heterologous infection which was characterized by increased immunopathology and decreased virus-specific memory CD8+ T cells. These studies demonstrate that progestins are a critical host factor mediating protection from severe influenza A virus by promoting tissue repair and reducing inflammation but can also make females more susceptible to secondary challenge with a heterologous influenza A virus by decreasing memory responses, and altering antibody responses. This suggests that progestin-based contraceptives may play complex, multifaceted roles during influenza A virus infection and vaccination.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
ADCC	antibody-dependent cellular cytotoxicity
AID	activation-induced cytidine deaminase
ARDS	acute respiratory distress syndrome
AREG	amphiregulin
ASCs	antibody-secreting cells
BAL	bronchoalveolar lavage
BMDCs	bone-marrow derived dendritic cells
COCs	combined oral contraceptives
COX-2	cyclooxygenase-2
Crs	respiratory system compliance
DC	dendritic cell
DFCO	diffusing factor for carbon monoxide
DMPA	depot medroxyprogesterone acetate
Dpi	days post-inoculation
EAE	experimental autoimmune encephalomyelitis
FSH	follicle stimulating hormone
HA	hemagglutinin
HIV	human immunodeficiency virus
HRT	hormonal replacement therapy
HSV	herpes simplex virus
IAV	influenza A virus
Ig	immunoglobulin
IL	interleukin
iNKT	inducible natural killer T
iNOS	inducible nitric oxide synthase
IUD	intrauterine device
LDH	lactate dehydrogenase
LH	luteinizing hormone
LNG	levonorgestrel
MALTs	mucosal-associated lymphoid tissues
MDCK	Madin-Darby canine kidney

MLD_{50}	mouse lethal dose 50
MPA	medroxyprogesterone acetate
NA	neuraminidase
NK	natural killer
NP	nucleoprotein
NO	nitric oxide
2',5'-OAS	2',5'-oligoadenylate synthase
P4	progesterone
PBMC	peripheral blood mononuclear cell
PKR	protein kinase R
PR	progesterone receptor
PRKO	progesterone receptor knock out
PRRs	pathogen recognition receptors
RIG-I	retinoic acid inducible gene-1
Rrs	respiratory system resistance
SA	sialic acid
SIV	simian immunodeficiency virus
STIs	sexually transmitted infections
ssRNA	single stranded RNA
TBI	traumatic brain injury
$TCID_{50}$	tissue culture infectious dose 50
TCM	T central memory
Th	T helper
TLR	toll-like receptor
TNF-α	tumor necrosis factor α
TRAIL	TNF-related apoptosis-inducing ligand
TRM	tissue resident memory
TSLP	thymic stromal lymphopoietin
WT	wild type

Chapter 1

INTRODUCTION

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In preparation for submission to *Mucosal Immunology* under the title: Progesterone-based therapies affect immune responses and susceptibility to infections at diverse mucosal sites

Introduction to progesterone

Natural progesterone (P4) is produced by the corpus luteum during the menstrual cycle in non-pregnant females and its production is sustained at high levels by the placenta during pregnancy (1). Serum levels of P4 fluctuate during the menstrual cycle with a peak of 20ng/ml during the luteal phase and the nadir (<1ng/ml) during the follicular phase (2). During pregnancy, the production of P4 is maintained by the placenta with increases from 12-90ng/ml during first trimester and up to 300ng/ml during the third trimester of pregnancy (2). In the 1940s synthetic analogs of P4, termed progestins, were developed and in the 1960s these compounds began being used as hormonal contraceptives (3). Exogenous P4 and progestins are used therapeutically in oral contraceptives by over 20 million young adult women in the United States alone (4).

Progestins and P4 can freely diffuse through the cell membrane and signal through the progesterone receptor (PR), located in cytoplasm, which when bound, translocates to the nucleus, and binds progesterone response elements to alter transcription and downstream signaling (5). Progesterone receptor signaling can also occur in a non-genomic signaling manner via the MAPK or PI3K/Akt pathway, for example, or through membrane-bound PRs that signal by either increasing cAMP or through the JNK pathway and bypass the classical PR pathway all together (6-9). Progesterone receptors have two isoforms, A and B, which are derived from the same gene (10). Progestins bind the PR with higher affinity than P4; with levonorgestrel (LNG), for example, binding the PR with a 300% affinity relative to P4 (11). These PR are present in a wide range of tissues, on a variety of different cell types, including immune cells such as natural killer (NK) cells, macrophages, dendritic cells (DCs), T cells and non-immune cells, such as epithelial cells, endothelial cells, and neuronal cells (12, 13). Progestins and P4 can also signal through other steroid receptors including the androgen, glucocorticoid, and mineralocorticoid receptors which, like all steroid receptors, are also present in the cytoplasm (14, 15). As each of these receptors are widely distributed throughout the body and can bind progestins and P4, the effects

of progestins and P4 are not limited to the reproductive tract but occur in the central nervous system, respiratory system, cardiovascular system, and in certain tumors (16, 17).

Progesterone-based contraceptives

As noted above, in addition to natural exposure during the menstrual cycle and pregnancy, women of reproductive ages can be exposed to P4 in the form of hormone-based contraceptives. The "pill" which was approved by the Food and Drug Agency (FDA) in 1960 is typically composed of a combination of progestins (**Table 1.1**) and estrogen, but many hormonal contraceptives, such as intrauterine devices, contain progestin only formulations (18). Progestins exert their reproductive effects by thickening the mucus, suppressing ovulation by altering the luteinizing hormone (LH) and follicle stimulating hormone (FSH) surges, and making the endometrium inhospitable for implantation (15, 19). Many different contraceptive formulations exist, with new generations of progestins being developed that have higher affinity to the PR thereby reducing the dose required to be efficacious as well as reducing potential side-effects (**Table 1.1**). These can be used in conjunction with estrogen in combined oral contraceptives (COCs), or alone in the form of implants, injections (usually with depot medroxyprogestone acetate (DMPA)), intrauterine devices, rings, or as emergency contraceptive (15). In the United States alone, 35% of adult women are currently using hormonal methods of contraception, and 88% of all adult women in the United States have been exposed to progestins in some form of contraceptives (20). Male hormonal contraception is currently being research and the administration of progestin such as LNG and etonorgestrel can also decrease pituitary LH and FSH production and testicular testosterone production and spermatogenesis without affecting secondary sexual characteristics (21).

Table 1.1: List of commonly used progestins

Progestin	Uses (reproductive system)
Progesterone (P4)	POC, HRT, dysmenorrhea treatment, ART
Norethindrone	COC, POC, endometriosis treatment, dysmenorrhea treatment
Depot medroxyprogesterone acetate (DMPA)	Injectable contraceptive, HRT
Levonorgestrel (LNG)	COC, IUD, emergency contraception, implants
Etonogestrel	Vaginal ring, implants
Drospirenone	COC, HRT

Key: POC = P4 only contraceptive, COC = combined oral contraceptives, IUD = intrauterine device, HRT = Hormonal replacement therapy, ART – assisted reproductive technology.
Sources: (15, 22, 23)

Progesterone-based therapies

In addition to their use in birth control, P4 and progestins are also used in combination with estrogen in hormone replacement therapy (HRT) in perimenopausal women to prevent the proliferative effects of estrogen on endometrial cells, which can lead to cancer and increased bone density (24). The potential side-effects of progestin-containing HRT, including increased breast cancer risks and heart disease, raised concerns following the Women's Health Initiative trial, but the study was conducted with medroxyprogesterone acetate (MPA), which can transactivate the androgen and glucocorticoid receptor and thus lead to increased undesired side-effects (25). Newer generations of progestins are now used to prevent these side-effects and reduce the risk of breast cancer (26).

Progesterone-based oral contraceptives have also been used for their "off-label" beneficial effects on conditions such as acne, polycystic ovary syndrome, and dysmenorrhea (4, 19). Progestins can also increase bone density and have been used to prevent osteoporosis (27, 28). More recently, P4 was shown to have a neuroprotective role following injury in the central

nervous system by dampening inflammation and promoting repair of myelin fibers in the context of traumatic brain injury and multiple sclerosis (17, 29). As such, it is currently used in clinical trials for treatment of traumatic brain injury and strokes in both men and women (29-32).

The Immunomodulatory Effects of Progesterone

Progesterone and immune cells

The presence of PR on a wide variety of immune cells, suggest that these cells might respond to P4 treatment and indeed, *in vitro* studies show that P4 can alter the immune environment in various tissues and cell culture systems by promoting an anti-inflammatory state (**Table 1.2**). Classical nuclear PR and membrane-bound PR are located in NK cells, macrophages, DCs, and T cells (12, 33-37).

Progesterone generally inhibits inflammatory innate immune responses. *In vitro* studies show that P4 can modulate immune pathway signaling in innate immune cells such as macrophages and DCs and suppress their activation (38, 39). For example, when P4 is bound to its receptor, it can directly interfere with the transcription factor NF-κB through transrepression and inhibit gene transcription downstream of the NF-κB pathway, including cyclooxygenase-2 (COX-2) to decrease inflammation (38, 40-42). In human primary myometrial cells, P4 inhibits the MAPK pathway and downstream COX-2 and IL-1β expression via the glucocorticoid receptor (43). Progesterone can also decrease inflammation by inhibiting the production of pro-inflammatory cytokines (e.g., TNF-α, IFN-γ, and IL-12) and increasing production of anti-inflammatory cytokines, including IL-10 (33, 39, 44, 45). Treatment of endometrial stem cell cultures, isolated from female patients with endometriosis, with different progestins reduces the production of IL-6, MCP-1, and TNF-α mRNA (46). Additionally, exposure of these stem cell cultures to either TNF-α or IL-1β decreases intracellular expression of PRs (44). Not all studies demonstrate anti-inflammatory effects of P4. For example, when cytokines in women taking

DMPA (see **Table 1.1**) were measured in endocervical swabs, levels of MIP-1, IL-6, and IL-8 were increased (47, 48). In addition to signaling through the PR, DMPA can signal through the androgen and glucocortoid receptors, which can result in effects that are different than P4 (25, 47).

In rodent bone-marrow derived DCs (BMDCs), administration of either P4 or LNG downregulates the activation of the toll-like receptors (TLR) 3 and 4 and the production of IL-6, IL-12p40, TNF-α, and IL-1β as well as the expression of CD80 and CD86 following stimulation with TLR ligands (33, 39). When BMDCs are co-cultured with T cells, the presence of P4 decreased T cell proliferation in response to LPS (33). Similarly, P4 treatment of the macrophage cell line RAW264.7 inhibits TLR3, TLR4, and TLR9 signaling in response to Poly I:C, LPS, or CpG DNA, respectively, and downstream IL-6 and nitric oxide production (41, 49). Treatment with P4 can also alter the phenotype of macrophages by inducing the expression of Fizz-1 and YM-1, two markers of alternatively activated macrophages, and decreasing the production of nitric oxide following LPS stimulation (50). In murine bone marrow-derived macrophages treated with LPS, P4 but not LNG decreases nitric oxide production and the ability to lyse *Leishmania* intracellular parasites (51). NK cells are susceptible to P4-induced cell death which can be blocked by treatment with the P4 antagonist, RU-486 (34). Eosinophil numbers in ovariectomized rats treated with P4 are increased as compared with ovariectomized rats (52).

Progesterone also alters the distribution and activity of T cells. Treatment of T cells *in vitro* with P4 can skew naïve T cells away from Th1 responses and towards a Th2 type response, with increased production of IL-4, IL-5 and IL-10 (45, 53, 54). In human monocyte-derived DCs, treatment with P4 induces a Th2 environment with increased concentrations of IL-10, IL-13, and IL-27 (45). In T cell lines, the addition of P4 to the culture media induces a Th2 environment, with greater secretion of IL-4 and IL-5 as compared with non-P4 treated cultures (53). Murine T cells cultured to differentiate into either Th1 or Th2 cells in the presence of P4, show a strong

bias towards Th2 with decreased IFN-γ production and enhanced Th2 responses, including production of IL-4 (54). *In vivo,* presence of P4 induces a regulatory milieu by increasing the production of the anti-inflammatory and repair cytokine TGF-β in the endometrium (55, 56). During pregnancy, high levels of P4 can shift the immune response towards a CD4+ T regulatory (Treg) phenotype (57). In human cord blood cells, P4 induces the differentiation of Tregs and suppresses Th17 cells through the modulation of transcriptions factors such as STAT5 and STAT3 (58).

In B cells, the effects of P4 treatment are less well characterized. *In vitro* studies with B cells and endometrial cell co-cultures from mice show that in the presence of P4, B cells have decreased expression of CD80 and CD86 and a limited ability to present antigen (59). B cell hybridomas treated with P4 show decreased cellular proliferation and antibody production (60). In murine splenic B cells, P4 treatment decreases activation-induced deaminase (AID) mRNA and the ability of these cells to undergo somatic hypermutation and class-switch recombination (61). In combination with estradiol, but not alone, P4 has the ability to suppress B cell lymphopoiesis in mice (62).

Taken together, these data illustrate that P4 and related compounds can bind to PRs to decrease inflammation by acting on the expression of cellular receptors, intracellular signaling and cytokine production to induce an anti-inflammatory milieu in a broad range of tissues.

Table 1.2: The effects of progestins on immunity

Immune Response	Progestin	Species	Outcome	References
TLR activity	P4, LNG	H, M	↓ TLR3, ↓ TLR4, ↓ TLR7, ↓ TLR9	(39, 41, 49, 63)
Type I IFNs	P4, MPA	H, R	↓ IFN-β	(33, 42, 64)
NF-κB signaling pathway	P4	H	Pathway inhibited ↓ COX-2	(38, 40, 43)
Macrophages	P4, LNG	M	↓ iNOS, ↓ NO ↑ Fizz-1, ↑ Ym-1	(49-51)
Dendritic cells	P4	H, M, R	↓ CD40, ↓ CD80, ↓ CD86 ↓ TNF-α, ↓ IL-6, ↓ IL-12, ↓ IL-1β ↑ IL-10,↑ IL-8 ↑ CD11c	(33, 39, 45, 65)
NK cells	P4	H	↓ IFN-γ ↑ Apoptosis (caspase-dependent)	(34)
Eosinophils	MPA	M	↑ Numbers	(66)
Pro-inflammatory cytokines	P4, MPA, DNG, NETA, LNG	H, M, R	↓ TNF-α, ↓ IFN-γ, ↓ IL-12, ↓ IL-6	(33, 41, 44, 51, 54, 60)
Anti-inflammatory cytokines	P4, MPA	H, M, P	↑ IL-4, ↑ IL-5, ↑ TGF-β, ↑ Il-13, = IL-10	(45, 53, 55, 56, 58, 66)
Chemokines	P4	M	↓ MIP-2, MCP-1	(67)
CD4+ Th1	P4	M, R	↓ Development	(33, 54)
CD4+ Th2	P4	H, M	↑ Development ↑ IL-4, ↑ IL-5, ↑ IL-13, ↑ IL-27 ↓ IFN-γ	(45, 53, 54)
Tregs	P4	H, M	↑ Percentages	(57, 58)
B cells	P4	M	↓ Expression CD80 and CD86 ↓ Proliferation ↓ AID and class-switching ↓ Antibody responses	(59-61, 68)

Key: P4= progesterone, MPA=medroxyprogesterone acetate, DNG=dienogest, NETA=noresthisterone acetate, H=human, M=mouse, P=primate, R=rat, TLR= toll-like receptor, COX-2=cyclooxygenase 2, iNOS= inducible nitric oxide synthase, NO=nitric oxide, AID=activation-induced cytidine deaminase.

Progesterone and non-infectious diseases

The immuno-modulatory effects of P4 result in profound effects of P4 on the outcome of diverse diseases. In mice sensitized with ovalbumin to model asthma, MPA increases allergic airway inflammation with increased eosinophilia and IL-5 but has no effect on the levels of IgE (66). In a murine model of multiple sclerosis (i.e., experimental autoimmune encephalomyelitis; EAE), administration of P4 decreases disease severity by reducing the production of pro-inflammatory cytokines, such as IL-1β and IL-17, limiting cellular infiltration into the central nervous system, and increasing IL-10 production (64, 67). In a mouse model of systemic lupus erythematosus, inducing glomerulonephritis, DMPA treatment reduces mortality and the levels Th1 autoantibodies leading to limited glomerular damage (69). In rats with spinal cord injuries, treatment with P4 attenuates the injury-induced expression of IL-1β, IL-6, TNF-α, iNOS, and COX-2 in the central nervous system and promotes proliferation of oligodendrocyte precursor cells in wild-type (WT) but not in PR knock out (PRKO) rats (70, 71). Progesterone treatment can reduce inflammation due to traumatic brain injury (TBI) in both adult and neonatal rats by reducing COX-2, prostanglandin E2, TNF-α, brain permeability, and edema (72, 73). Human clinical studies also show that administration of P4 to patients with acute severe TBI improves neurological outcomes (32). When tested in Phase 3 clinical trials, P4 treatment failed to reproduce these findings but it has been suggested this may be due to suboptimal dosage and treatment duration along with the complexity of TBI pathophysiology and Phase 2B clinical trials are currently underway (31, 74, 75). In addition to studies in TBI models, the role of P4 in cerebral ischemia has been thoroughly studied, primarily in male rats, but also to a lesser extent in female rats as well as aged animals. Animals administered P4 show improved motor functions as well as decreased infarct volume and neuronal loss, with P4 upregulating VEGF and MMP9 and decreasing inflammation (76-82). Taken together these studies illustrate that P4, by decreasing inflammation and pro-inflammatory cytokines, limits non-infectious diseases caused by excessive

inflammation, such as some autoimmune diseases, and improves the outcome of brain injuries and stoke by promoting repair.

The Effects of Progestins on Infectious Diseases at Mucosal Sites

Background

Mucosal sites are characterized by the presence of a mucous membrane composed of epithelial cells that provide both a physical barrier and early production of innate immune defenses, including mucous and antimicrobial secretions. The epithelia also contains local mucosal-associated lymphoid tissues (MALTs) that resemble lymph nodes and serve as the inductive sites for the adaptive immune response (83). Mucosal sites are present throughout the body and include the respiratory tract, the gastrointestinal tract, the female reproductive tract, the eyes and the nose. They are particular vulnerable to infections as they come in contact with the exterior of the body and as such, form the main route of entry for infectious agents invading the host (**Table 1.3**) (84). In additional to being exposed to pathogens, mucosal sites are continuously exposed to commensal bacteria which need to induce tolerance mechanisms rather than initiate an immune response to avoid excessive and constant inflammation (85). Induction of a mucosal immune response generally occurs at inductive sites where antigen is captured by antigen presenting cells that can carry the antigen via the lymphatics to the local draining lymph node where it can be presented to naïve T and B cells and initiate adaptive immune responses. These activated T and B cells then need to traffic back through the bloodstream to the local mucosal sites, directed by homing signals including $\alpha_4\beta_7$ and CCR9 (86, 87). Production of IgA at the sites of infection involves the production of the polymeric IgA receptor by epithelial cells which allows for transport of IgA across the mucosal barrier and the generation of an IgA dimer (88, 89). Similarities between the different mucosal sites, termed "the common mucosal immune system" involve the homing of antigen-specific cells to a mucosal effector site different than where the antigen was first sensed (90). This is important in vaccination contexts where oral

vaccination can also protect against infections at other sites than the gastrointestinal tract (91). In this chapter, I will be describing the role of progestins at various mucosal sites which can be found summarized in **Table 1.3**. Despite these mucosal sites being anatomically distinct compartments, the similarities in mucosal immune responses may allow for us to draw parallels between the responses that occur at different sites.

Urogenital tract

Epidemiological studies show that when compared with males, females have higher rates of sexually transmitted infections (STIs), including human immunodeficiency virus (HIV), herpes simplex virus (HSV), gonorrhea, and chlamydia. The different stages of the menstrual cycle along with the use of hormonal contraceptives have been shown in both human and animal models to increase susceptibility to STIs (92, 93) and progestins have been implicated as a factor that may contribute to this heightened susceptibility (94-97). Many reasons have been proposed for these negative effects of progestins on STIs including thinning of the vaginal epithelium (98, 99), increases in virus entry co-receptors (100-102), and induction of an anti-inflammatory cytokine milieu lacking essential anti-microbial, pro-inflammatory cytokines such as IFN-γ and TNF-α (97, 101).

The effects of progestins have been extensively studied in the context of HIV, where multiple epidemiological studies, *in vivo* studies in non-human primates, and *in vitro* studies have assessed the effects of progestins on the outcome of HIV acquisition, transmission, and virus replication. In both humans and non-human primates, the risk of HIV acquisition is increased in females during the luteal phase of the menstrual cycle, when P4 is at its highest levels, and following use of hormonal contraceptives containing progestins (103-106). In non-human primates, there is an increased risk of simian immunodeficiency virus (SIV), and higher viral loads following P4 treatment (97, 107). In macaques treated with DMPA, acute viremia and

transmission of multiple viral variants is increased concomitantly with a decrease in virus-specific IFN-γ production (97). In women taking DMPA, levels of the chemokine CCL5 are significantly increased in cervical swabs, but concentrations of pro-inflammatory cytokines such as IL-1β or IL-6 remain similar to women not taking contraceptives (48). *In vitro* data show that treatment of PBMC with P4 leads to increased expression of the HIV receptor CXCR4 but not CCR5 in both HIV infected and non-infected women (102). Genital epithelial cells also have a higher uptake of HIV by endocytosis when exposed to P4 and to a greater extent with MPA (108).

Huijbregst et al. studied extensively the effects of either P4 or MPA in human peripheral blood mononuclear cells (PBMCs) and cervical lavages. They found that upon activation with cytokine-coated activating beads, PBMCs treated with MPA had reduced IFN-γ, IL-6, IL-12, IL-17 and TNF-α production, and both MPA and P4 reduced IL-2 and IL-4 production (101). When these PBMCs were treated with P4 and infected with HIV, MPA but not P4 increased viral titers (101). Additionally, cervicolavage fluid from women taking DMPA has decreased concentrations of IFN-α as compared with concentrations in women not taking any form of contraceptive, which mirrors the suppressive effects of progestins on type I IFN production by plasmacytoid DCs following stimulation with TLR ligands (101).

Many of the epidemiological studies of progestins and HIV focus on women using DMPA, which may increase the risk of HIV acquisition, but more recent meta-analysis studies found that overall progestin-based hormonal contraceptives do not increase the risk of HIV acquisition or disease progression (104, 109-112). It could be that DMPA promotes HIV acquisition via a modulation of the immune response through the glucocorticoid but not the progesterone receptor signaling. Progestins can have their biological effects through either the glucocorticoid or progesterone receptor and all progestins bind to these receptors with different affinities (14).

Herpes simplex virus (HSV) is the most commonly transmitted STIs and progestins can increase susceptibility and decrease protection during HSV vaccination (92, 95, 96, 113-116). Studies in ovariectomized mice show that DMPA leads to HSV-1 reactivation, with a decrease in numbers of virus-specific neuronal CD8+ T cells and their ability to control the virus including their lytic capacity and production of the antiviral cytokines IFN-γ and TNF-α (95). DMPA also impairs the CD8+ T cell response by decreasing the expression of co-stimulatory molecules on DCs (117). In mice, treatment with either DMPA, P4, or LNG increased susceptibility to HSV-2 by increasing levels of IL-1β and inflammation and enhancing mucosal permeability to facilitate entry of inflammatory cells by reducing the expression of the cadherin desmoglein-1a (116, 118, 119). Human primary genital epithelial cells are highly susceptible to HSV-2 infection but unlike murine models, P4 treatment is protective and limits virus shedding as compared to control-treated cultures (120). In the context of HSV-2 vaccination, DMPA and P4 decrease anti-HSV-2 IgG and IgA in vaginal washes, increase viral shedding, and fail to protect mice following HSV-2 challenge as compared to estradiol-treated mice (113, 118, 119).

In rats, P4 increases susceptibility to the bacterium *Chlamydia trichomatis* by increasing inflammation, production of IFN-γ and IL-10, and pathogen load (94, 121). In women using DMPA, susceptibility to infection with *Neisseria gonorrhea* is significantly decreased (122), but treatment of primary human cervical cells with P4 increases the ability of the bacteria to survive by increasing AKT activity and iNOS production (123). When P4-treated female mice are infected with *N. gonorrhea*, bacterial loads and duration of infection are increased with increasing levels of thymic stromal lymphopoietin (TSLP) and Tregs and decreasing Th17 cell responses (124). Vaginosis can be caused by many pathogens, the most common being *Candida albicans*; P4 and progestin-based hormonal contraceptives have all been shown to be beneficial by reducing prevalence and incidence of vaginitis (125). *In vitro* studies show that P4 can decrease *C. albicans* biofilm formation thus preventing colonization and invasion of the vaginal mucosa

(126). Taken together these data show that the anti-inflammatory properties of progestins may lead to increased susceptibility to STIs, but the type of progestin may affect the risk of acquisition or disease progression differentially.

Gastrointestinal Tract

A limited number of studies have accessed the role of progestins in the gastrointestinal tract, with focus being on either bacteria or parasitic infections. In the context of murine infection with *Salmonella typhimurium,* the causative agent of typhoid, P4 treatment increases host survival by increasing cellular infiltration and promoting clearance of the bacteria (127). Similarly, following infection of hamsters with the parasite, *Taenia solium,* P4 increases host survival and decreases tapeworm length by recruiting mast cells and increasing levels of the pro-inflammatory IL-4, IL-6, and TNF-α in the intestinal mucosa which can promote parasite elimination (128). In the context of gastritis due to *Helicobacter pylori* infections, P4 reduces gastrointestinal inflammation in ovariectomized gerbils and reduces the effects of gastritis (129). In summary, progestins may be protective against gastrointestinal infections by increasing host survival through their immunomodulatory effects.

Respiratory Tract

To date, only two studies have assessed the role of progesterone or progestins on infectious diseases in the respiratory tract. The first study looked at the effect of DMPA on the outcome of a *Mycobacterium tuberculosis* infection and found that DMPA can increase the viral load by inducing an anti-inflammatory environment with a decrease in the production of TNF-α, IFN-γ, and G-CSF (130). The second study is from this thesis, in which administration of P4 to ovariectomized mice prevented severe outcome from influenza A virus infection by decreasing inflammation and promoting repair of the pulmonary tissues through the epithelial growth factor amphiregulin (AREG), which is the focus of the work described in chapter 1 (131). Antibody

titers and memory CD8+ T cell responses are also decreased following treatment of ovary-intact mice with either P4 or LNG, which results in greater susceptibility to a challenge with a heterologous virus and will be the focus of chapter 2 (132).

These results show that progestin can reduce inflammation in the respiratory tract, which is detrimental in the context of tuberculosis, but beneficial during influenza A virus infection. This discrepancy may depend on whether control of severe disease requires a strong pro-inflammatory response to clear the pathogen (e.g., tuberculosis) or causes excessive inflammation leading to tissue damage (e.g., influenza).

Table 1.3: The effects of progestins on viral, bacterial, and parasitic infections of mucosal sites

Pathogen	Site	Progestin	Outcome of disease	Immunomodulatory effects	References
Human immunodeficiency virus (HIV)	Human female reproductive tract	DMPA	↑ Risk acquisition and transmission ↑ Virus uptake by genital epithelial cells ↓ Risk of acquisition in some human studies	↓ Innate and adaptive immunity ↓ IFN-γ, TNF-α, IL-2, IL-4, IL-6 IL-12, MIP-1α, TNF-α, ↓ IFN-α ↓ IFN-α and TNF-α production in pDCs ↓ CCR5 and CXCR4	(101, 104, 108, 109, 111)
		P4	↓ HIV replication in human trophoblasts	↑ Presence of CXCR4 ↑ or ↓ CCR5 ↓ TNF-α, IL-2 and IL-4	(100, 102, 133)
		Net-EN	No significant risk increase	N/A	(110, 111)
Simian immunodeficiency virus (SIV)	Primate female reproductive tract	P4	↑ Transmission ↑ Viral Loads	↓ Epithelium lining	(107)
		DMPA	↑ Susceptibility ↑ Acquisition and shedding	↓ IFN-γ secreting CD8 T cells	(97)
Herpes Simplex Virus type 1 (HSV-1)	Rodent female reproductive tract	DMPA	↑ Virus reactivation	↓ Virus-specific CD8 T cells ↓ IFN-γ and TNF-α ↓ Granzyme B and lytic granules ↓ Macrophages, NK and NKT cells ↓ CD40 and CD80 expression on DCs	(95, 117)
Herpes simplex virus type 2 (HSV-2)	Rodent female reproductive tract	DMPA	↑ Susceptibility ↑ Virus shedding ↑ Pathogenesis ↓ Protection of HSV-2 vaccine	↓ IgG and IgA levels ↑ IL-1β, CXCL7, CXCR1 ↑ IL-10 ↑ Mucosal permeability	(96, 116, 118, 119)
		P4	↓ Protection of vaccine against challenge ↓ Virus shedding	↑ Neutrophil infiltration ↑ CCL5, CXCL2, CCL2, CXCL10	(113, 114, 120)
		LNG	↑ Susceptibility	↑ IL-1β	(116)

Chlamydia trichomatis (Chlamydia)	Rodent female reproductive tract	P4	↑ Susceptibility ↑ Pathogen load	↑ Inflammation ↑ IL-10 and IFN-γ	(94, 121)
Neisseria gonorrhoeae (Gonorrhea)	Mammalian female reproductive tract	P4	↑ Bacterial load in primary human cervical epithelial cells ↑ Length of infection in mice	↑ TSLP ↑ Regulatory T cells ↓ Th17 cells ↑ Production of nitric oxide	(123, 124)
		DMPA	↓ Rates infection in women	N/A	(122)
Gardnerella vaginalis (vaginosis)	Human female reproductive tract	DMPA or Net-EN	↓Prevalence and incidence	N/A	(125)
Candida albicans (Candidiasis)	Human female reproductive tract	P4	↓ Biofilm formation, colonization and invasion	N/A	(126)
Salmonella typhimurium (Typhoid)	Rodent gastrointestinal tract	P4	↑ Survival of the host ↑ Resistance ↑ Clearance of bacteria	↑ Cellular infiltration	(127)
Helicobacter pylori (Gastritis)	Rodent gastrointestinal tract	P4	↓ Incidence of gastritis	N/A	(129)
Taenia solium (Taeniosis)	Rodent gastrointestinal tract	P4	↑Host survival by increasing inflammation ↓ Parasite load	↑ Proliferation of parasite-specific leukocytes ↑ IL-4, IL-6 and TNF-α	(128)
Mycobacterium tuberculosis	Rodent respiratory tract	DMPA	↑ Bacterial load	↓ TNF-α, G-CSF, IL-10 and IFN-γ	(130)
Influenza A viruses (Flu)	Rodent respiratory tract	P4, LNG	↓ Pathogenesis and severe outcome ↑ Host survival ↑ Repair and pulmonary function	↑ TGF-β, IL-6, IL-22 ↑ Amphiregulin ↑ Th17 cells	(131)

Key: P4 = progesterone, DMPA = depot medroxyprogesterone acetate, LNG = levonorgestrel, Net-EN = norethisterone oenanthate, N/A= not available

Influenza A virus

Background

Influenza A viruses (IAV), along with influenza B, C and D are part of the *Orthomyxoviridae* family, with subtype A being the main cause of zoonotic and human infections (134, 135). Influenza viruses are enveloped segmented single stranded RNA (ssRNA) viruses and the surface of the virion is composed of the hemagglutinin (HA) and neuraminidase (NA), which define the different influenza A virus subtypes (134). A combination of 18 different HA and 9 different NA subtypes have been found in circulating influenza A viruses (136). IAVs can also be further characterized based on their antigenic properties and as such, HA proteins are further divided into two phylogenetic groups: group 1 which includes H1, H5 and H9 and group 2 that contains H3 and H7 for example (136). Along with the HA and NA, the matrix 2 (M2) protein is also part of the envelope. The matrix protein 1 (M1) lies just beneath the envelope, and the core of the virus is composed of the ribonucleoprotein complex (which contains the viral RNA) which is encapsidated by the nucleoprotein (NP), the polymerases (PA, PB1 and PB2), and the nuclear export protein NS2 (134).

Influenza A viruses are found mainly in waterfowl which are the primary reservoir, but these viruses also circulate in birds, swine, humans and other mammals (e.g., bats, cattle and seals), and can be transmitted across species (137, 138). Host specificity as well as tissue tropism is determined by the binding of the HA to sialic acid (SA) on the surface of epithelial cells. For example, human IAVs HA bind preferentially to α2,6-Gal terminated SA, avian IAVs HA binds to α2,3-Gal terminated SA, and swine IAVs HA bind to both SAs (135, 139). The sporadic emergence of a new human-adapted IAV from a zoonotic virus is usually the cause of worldwide pandemics such as the 1918 Spanish flu (H1N1), 1957 Asian flu (H2N2), the 1968 Hong Kong flu (H3N2) and the 2009 H1N1 pandemic (135). Aside from the severe impact of these sporadic pandemics, seasonal influenza infections cause over 200,000 hospitalizations and up to 36,000

deaths each year in the United States alone (140). The economic impact of IAV infection is also very important with an estimated 8 billion dollars in loss not only due to hospitalizations but also projected lost earnings in the United States (141). Currently, there are two IAV subtypes circulating in humans: the H1N1 and H3N2 viruses as well as either one or two influenza B viruses and all are contained in the annual seasonal influenza vaccine (142). New IAV variants also emerge from point mutations in the HA or NA that occur during virus replication through a process termed antigenic drift, as these enable the virus to evade the host immune response despite pre-existing antibodies to the original strain (143, 144). For example, circulating viruses from the 2009 H1N1 pandemic acquired a K166Q mutation in the HA during the 2012-2013 influenza season and this antigenic variant is now present in 99% of all pandemic H1N1 isolates (145).

Innate immune responses to influenza A viruses

The first line of defense against any respiratory pathogens is formed by the barrier provided by the epithelial cells that line the respiratory tract which includes the nose, trachea and lungs, along with the mucus and other antimicrobial compounds they produce (146). Aside from being the first barrier that the viruses encounter, epithelial cells are also the primary cell type that IAV can infect, and replicate in (147). This leads to lysis of these virus-infected epithelial cells either directly by the virus itself or from targeting by immune cells, which induces the destruction of the pulmonary epithelium (143). Influenza A viruses can cross the epithelial barrier and be sensed by antigen-presenting cells, such as macrophages and DCs, which can initiate an adaptive immune response by presenting viral antigens to T cells (148, 149).

Tissue-resident alveolar macrophages are another line of defense against IAVs and promote viral clearance through phagocytosis of either opsonized viral particles or virus-infected apoptotic cells. During steady-state conditions, alveolar macrophages help maintain lung homeostasis; following IAV infection, these cells polarize towards an M1 inflammatory

phenotype and produce TNF-α and iNOS and contribute to viral clearance (150, 151). Depletion of M1 macrophages by deleting CCR2+ cells reduces the severity of IAV infection with decreased production of IL-6, TNF-α and CCL2 (152). Paradoxically, depletion of alveolar macrophages by clodronate liposomes prior to IAV infection leads to increased mortality by respiratory failure and uncontrolled viral replication with reduced numbers of virus-specific CD8+ T cells (153-157). This demonstrates that alveolar macrophages are essential during IAV infection but in excess can also lead to alveolar injury. Similarly, depletion of neutrophils leads to increased morbidity and mortality despite the fact that exacerbated IAV pathology is linked to increased neutrophil infiltration (153, 158, 159). Neutrophils are necessary for virus clearance and control of inflammation as studies depleting neutrophils in mice have shown increased mortality, virus titers, and pulmonary immunopathology (160, 161). However, like macrophages, excessive infiltration of neutrophils can be deleterious, therefore neutrophils are necessary but in excess can also cause immunopathology (153, 160, 161).

Lung resident DCs play an important role during IAV infection and can acquire antigen either directly by infection with IAV, or by phagocytosis of virus-infected epithelial cells, and have been shown to be better antigen-presenting cells than macrophages (148, 162). CD103+ DCs reside in the pulmonary epithelium, sample virus particles in the airway lumen, and can migrate to the lymph nodes to present viral antigen and activate naïve CD4+ and CD8+ T cells by cross-presentation (163, 164). TNF-α and nitric oxide producing DCs (TiP DCs) are increased in the lungs following infection highly pathogenic IAV strains and also play a role in the priming of virus-specific CD8+ T cells (165). Dendritic cells can also control IAV infections and contribute to excessive pathology by the production of pro-inflammatory cytokines including IL-6, TNF-α, and type I IFNs (148). The sensing of IAV in epithelial and antigen-presenting cells occurs through PRRs, including TLRs and RIG-I-like receptors (RLRs). For example, TLR7 senses ssRNA in the endosomes when the ribonucleoprotein is release from the IAV particle (166). At

later stages of infection, the retinoic acid inducible gene-1 (RIG-I) receptors recognizes the uncapped 5'triphosphate RNA of the virus in the cytosol (166). These PRR pathways lead to the activation of either the NK-κB or IRF3/7 pathways to induce the production of pro-inflammatory cytokines including TNF-α, IL-6, type I and type III IFNs and chemokines, such as CCL2 and CCL3 (148, 167, 168). IAV infection also triggers the inflammasome pathway through either an M2-induced proton flux, or sensing of ssRNA, and leads to the production of IL-1β and IL-18 (169, 170). Additional antiviral sensors, triggered and amplified by type I IFN signaling, are key in the immune response to IAV, including the RNA-dependent protein kinase R (PKR) which limits viral replication by blocking cellular translation, and 2',5'-oligoadenylate synthase (2',5'-OAS) that activates RNAse L and degrades viral and cellular RNA leading to apoptosis of the virus-infected cell (171, 172). IAVs have evolved a multitude of evasion mechanisms that act at many different levels of these PRR pathways, including the NS1A protein that inhibits RIG-I by preventing oligomerization of the CARD domain of RIG-I (171, 173). The production of these pro-inflammatory cytokines and chemokines in the lungs during IAV infection can lead to the recruitment of cells from the periphery and activation of local resident cells to produce a pro-inflammatory environment and promote viral clearance (148). However, in excess these pro-inflammatory responses can lead to extensive pulmonary tissue damage and these responses need to be balanced to avoid immunopathology while allowing virus clearance. Innate immune responses to IAV are not sufficient to resolve IAV infection and require the help of the adaptive immune responses with a combination of cytotoxic CD8+ T cells, CD4+ T cells and production of antibodies by B cells at the site of infection (149).

Adaptive immune response to influenza A viruses

Protective adaptive immune responses to IAV infections, include antibodies and T cell responses. Protection during primary IAV infection is mediated by cellular immune responses including macrophages and T cells and prevention of re-infection is correlated with anti-influenza

specific antibodies and as such are a relative correlate of protection. Antibodies recognize the envelope proteins HA and NA, which are the immunodominant proteins of the virus, generate long-lasting protective immunity by preventing virus attachment and entry (for antibodies against HA) and limit viral spread (for antibodies against NA) (174-176). Most antibodies against the HA bind the highly variable globular head domain of the protein which is in proximity of the receptor-binding domain, and only confer strain-specific protection (177). More recently, broadly neutralizing HA antibodies targeting the conserved stalk domain have been discovered in mice, humans and ferrets, and mediate cross-protection against IAV within the same HA phylogenetic groups (178-182). Antibodies against the internal NP protein are generated later during infection are non-neutralizing and promote protection through antibody-dependent cellular cytotoxicity (ADCC) (183). Human studies show the presence of both virus-specific IgA and IgG antibodies in nasal washes following IAV infection; IgG alone, however, is not sufficient to reduce viral shedding in the nasal cavity. Secretory IgA-mediated protection more than IgG provides protection against IAVs in the lungs (184-186).

Naïve CD4+ T cells differentiate into different Th subsets following IAV infection and support the formation of both virus-specific CD8+ T cells and antibody-secreting B cells. Th1 cells, which produce IFN-γ and IL-2, are involved in promoting cytotoxic CD8+ T cells which control IAV replication, and help with the promotion of B cell isotype switching from IgG1 to IgG2a or IgG2b, which induce a better protection during IAV infection (187-190). Th17 cells play a protective role in the immune response to IAV through the production of IL-17 which leads to an increase in antimicrobial peptides, proliferation of lung epithelial cells, and recruitment of neutrophils (191-193). Memory CD4+ T cells play a role in heterosubtypic responses to IAV by enhancing protection through the secretion of IFN-γ and faster virus clearance independent of CD8+ T cell responses (194). Additionally, virus-specific memory

CD4+ regulatory T cells actively recruited to the lungs following a secondary IAV infection and control the proliferation of memory CD8+ T cells to dampen inflammation (195).

Cytotoxic CD8+ T cells control IAV infection by producing antiviral cytokines, including IFN-γ and TNF-α, and lysing virus-infected cells (196, 197). CD8+ T cells can target virus-infected cells for lysis in three different ways:1) in a granzyme- and perforin- dependent manner, 2) using the Fas/FasL pathway, and 3) via the TNF-related apoptosis-inducing ligand (TRAIL) pathway (197, 198). Virus-specific CD8+ T cells alone are capable of clearing IAV infections, independent of neutralizing antibodies (197, 199). CD8+ T cells recognize epitopes derived from internal proteins, and studies using *ex vivo* stimulation of human PBMCs have shown that peptides from the M1 and NP proteins are the major targets for T cell immunity against IAV (200-202). Memory CD8+ T cells have the ability to target a broad range of peptides derived from IAV proteins that can be relatively well conserved, such as the nucleoprotein to provide immunity against a broad range of distinct IAV strains and induce heterologous immunity against novel IAV strains (180, 203-205). In addition to circulating memory T cells, and central memory T cells which traffic through the lymph nodes, the role of tissue-resident memory cells in promoting a local and immediate protection in the lungs during IAV infection with the ability to expand rapidly, kill virus-infected cells, recruit circulating memory T cells, release cytokines has been shown to be indispensable in generating both homotypic and heterosubtypic protection (206-208). Other adaptive immune cell types, including inducible natural killer T (iNKT) cells have a protective role during IAV pathogenesis, through the production of IL-22 which leads to repair and protection of the epithelial barrier (209).

Risk factors for severe influenza

A number of host factors contribute to IAV severity including sex, age, obesity, pregnancy, asthma, and the presence of co-morbidities (210-215). Females generally mount

higher immune responses than males with greater antibody and cellular immune responses and reduced susceptibility and viral loads following viral infection (211, 216, 217). However, during infection with viruses such as IAV, in which severe disease is caused by immunopathology, this heightened immunity may be unfavorable. In both human and murine models of IAV, females are more likely to develop severe disease than males (214, 218, 219). During the 2009 H1N1 pandemic for example, in adults (20-70 years of age), females had higher hospitalization rates, longer length of stay and increased mortality as compare to males (220, 221). Adult female mice inoculated either with H1N1, H3N2, or H7N9 viruses have higher morbidity and mortality than males (218, 219, 222). Females have higher levels of pulmonary pro-inflammatory cytokines and chemokines including TNF-α, IFN-γ, IL-6, and CCL2 than males during the acute phase of H1N1 infection (222). Females infected with an H3N2 virus have higher levels of IFN-γ and MCP-1 during the acute phase of infection and impaired lung function than males (223). Similarly, females infected with an H7N9 virus mounted higher cytokine responses than males, including increased levels of IL-6, IL-9, TNF-α, MCP-1 and CCL-5 during the acute phase of infection (219). All three of these studies show no differences in viral titers between the sexes which suggests that it is the host immune response and not the virus which mediates the observed sex differences in morbidity and mortality (219, 222, 223). However, females sex steroid hormones alone do not explain these differences as both estrogens (E2 specifically) and P4 decrease pro-inflammatory responses, including TNF-α and CCL2 during IAV infection (131, 158, 222).

Pregnant women are 3 to 10 times more likely to be hospitalized, and are at greater risk of intensive care unit (ICU) admission and secondary bacterial infections as compared to age-matched non-pregnant females (224-226). During the 2009 H1N1 pandemic for example, pregnant women were at greater risk of a severe outcome, with approximately 80% of maternal deaths occurring during the third trimester of pregnancy (227-230) Although the outcome of seasonal influenza virus infection is less severe than the outcome of pandemic influenza viruses,

pregnant women have a longer length of hospitalization from infection with seasonal influenza viruses (231). This increased susceptibility to IAV may be due to changes in the immune status during pregnancy where the local and systemic environment shift towards an anti-inflammatory milieu in order to sustain a healthy pregnancy and avoid fetus rejection. This shift towards an anti-inflammatory environment is characterized by an increase in CD4+ Th2 and Tregs (232-236). Studies looking at IAV during pregnancy show a decrease in plasmacytoid DCs leading to a downregulation of cytotoxic CD8+ T cells and IFN-γ production in humans and mice (237). In a pregnant mouse model, IAV increased numbers of pulmonary macrophages and Tregs as compared to non-pregnant mice (213). Pregnant mice infected with ma2009 H1N1 have greater mortality and production of pro-inflammatory cytokine and chemokines in the lungs, including TNF-α, CCL2, CCL3, CXCL1, as compared with non-pregnant female mice (213, 238, 239). Whether control of virus replication is diminished during pregnancy is not clear because data regarding differences in viral loads are contradictory (213, 238). These immune changes parallel increases in sex steroid hormones, including estrogens and progesterone as well as anatomical and physiological changes which increase cardiopulmonary demands and all of these changes may contribute to an increased risk of severe IAV outcome during pregnancy (225, 240-242).

Similarly to pregnant individuals, obesity is also associated with respiratory complications and higher hospitalization rates during infection with seasonal and pandemic IAVs (215). Studies in mice have shown that both diet-induced and genetically obese mice have increase morbidity with severe lung pathology associated with impaired pulmonary wound repair, higher viral titers and dysregulated memory CD8+ T cells responses (212, 243, 244). Studies in both mice and humans also show that obesity impacts antibody responses and lowers responsiveness to influenza vaccination (244-246). This impaired response to IAV in obese subjects is thought to be a combination of immune dysregulation and decreased lung function (215, 243).

Animal models for influenza A virus

In order to better understand IAV pathogenesis, risk factors, vaccine responses and antiviral efficacy, a variety of animal models are commonly used, with mice and ferrets being the preferred models (247, 248). Mice are the most widely used models as they are practical, small, relatively inexpensive, and develop similar immune responses, despite the lack of IAV transmission and different clinical presentation than humans (247, 248). Clinical signs of IAV infection in mice include: weight loss, hypothermia, ruffled fur, lethargia, huddling, and dyspnea (131, 158, 222). Viruses generally need to be mouse-adapted, except for highly pathogenic strains, as mice and humans both have α2,3-linked SA in ciliated and type II alveolar epithelial cells but the α2,6-linked SA, which is the preferred receptor for human viruses is not present in mice (249). Influenza virus are mouse-adapted through serial passage in mice to increase receptor binding and virulence by generating amino acid mutations (250, 251). Inbred mice lack the Mx1 gene, which makes them more susceptible to IAVs, unlike wild mice (252, 253).

Ferrets have been used in influenza research since the 1930s and represent a more accurate animal model of IAV transmission, have similar clinical symptoms to humans, and do not require prior virus adaptation due to similarities in their sialic acid distribution (254-259). They are widely used to determine antigenicity of IAV vaccines and for testing of new antiviral drugs (254, 257, 260). Guinea pigs are another animal model that support IAV transmission but lack clinical signs of disease (247). Syrian hamsters were used in the 1960s and 1980s to analyze antibody responses to IAV but do not transmit IAVs (247). Pigs are a natural reservoir for IAV but lack practical aspects for animal husbandry and are mainly used in the development of swine IAV vaccine and have the advantage of supporting the infection of most IAV subtypes (261). Non-human primates are rarely used but are also susceptible to human IAVs and are used in vaccination studies (247, 248, 262). Despite differences in clinical symptoms, transmission and immune responses between animal models and humans, they serve as a good surrogate and allow

to investigate different aspects of IAV infection, pathogenesis, transmission and vaccination. The greatest caveat with all these models is that they are influenza naïve and studies have shown that humans by age six have all been exposed to IAVs which is something that needs to be considered in animal models of IAV (263).

Influenza A virus pathogenesis

Influenza A viruses cause an acute respiratory infection typically characterized by virus replication in respiratory epithelial cells, initiation of a 'cytokine storm' with production of IL-1β, TNF-α, IFN-α, IL-6 and CCL2 in the host, and long-term damage to pulmonary tissues (264, 265). This pulmonary inflammation is characterized by edema, bronchitis, lung fibrosis, diffuse alveolar damage, infiltration of immune cells and death of epithelial cells which threatens proper gas exchange and pulmonary function (153, 265-268).The typical presentation of influenza in adults and children is an abrupt onset of fever and chills along with headaches, sore throat and myalgia (143). In the most severe cases of IAV, excessive pulmonary infiltrates and hypoxemia can lead to acute respiratory distress syndrome (ARDS) which accounts for 4% of all hospitalization for respiratory failure during influenza infections (269, 270). Long after the symptoms of disease have subsided, pulmonary tissue damage can persist and increase the risk of secondary bacterial infections (265, 271). The 2009 H1N1 pandemic, in particular, was associated with increased inflammation and pulmonary tissue damage requiring extensive tissue repair (272). Protection requires a balance between inflammatory responses generated to clear the virus and those that repair the damage cause by cellular infiltration and the local protein mediators. Repair of damaged tissue involves a complex interplay among many cells, cytokines, chemokines, growth factors, and extracellular matrix proteins that serve to remodel tissue (273, 274). Repair of the damage induced in the lungs following IAV infection is generally orchestrated by macrophages, Tregs, and associated cytokines, including IL-10, IL-22 and TGF-β, along with remodeling by epithelial cells (275). Recent studies in both gastrointestinal and respiratory

mucosa have demonstrated a crucial role for IL-22 as a gatekeeper to promote epithelial integrity by promoting cellular proliferation and production of antimicrobial peptides (276-278). During IAV infection, IL-22 is essential for promoting repair of damaged epithelial and recovery to a healthy lung function. This was demonstrated in IL-22 KO mice infected with H1N1, where the lack of IL-22 led to increased morbidity, worsened lung injury, and increased edema without affecting pulmonary virus titers as compared to wild-type mice (277). In addition to cytokines, damaged epithelial cells release factors, including the epidermal growth factor-like molecule, amphiregulin (AREG) that promote repair and remodeling of lung tissue damaged during IAV infection (279, 280). AREG is involved in pulmonary tissue remodeling and repair during many different aspects of lung pathology including lung injury, asthma, and infection (273, 279-286). During IAV infection, administration of recombinant AREG protects mice from severe IAV infection by decreasing hypothermia, improving pulmonary function, and decreasing protein leakage into the airways (131, 279, 280). AREG is produced primarily by epithelial cells (131, 287), but type 2 innate lymphoid cells (ILC2) and Tregs have also been shown to produce AREG during IAV infection and contribute to the repair during resolution of infection (279, 280, 287, 288).

Taken together, these data suggest that the clearance and resolution of IAV infection relies on a delicate balance of pro- and anti- inflammatory mediators with any dysregulation towards exacerbated inflammation leading towards severe influenza illness and sequelae to the lungs. I hypothesize that given its anti-inflammatory role and repair role, P4 may help tilt the balance towards a dampening of inflammation, thus protecting the respiratory epithelium while inducing repair, without compromising the immune response necessary to clear the virus.

Specific Aim 1

Progesterone has immunomodulatory and reparative properties in the reproductive tract and in the central nervous system, that have yet to be evaluated in in the respiratory tract. I hypothesized that administration of progesterone to progesterone-depleted female mice would impact the outcome of influenza A virus infection by dampening inflammation and promoting pulmonary repair. Specifically, the dampening of the exacerbated inflammation by progesterone during lethal influenza A virus infection would protect these mice from succumbing to the infection, and promote production of cytokines, such as TGF-β and IL-22, that lead to the repair of epithelial cells without impacting virus replication.

To further assess the role of progesterone in repair, I used a non-lethal influenza A virus model to evaluate the effects of progesterone during the recovery phase and I hypothesize that administration of progesterone would both dampen the inflammation during the peak of infection, and promote repair during the recovery phase to hasten the repair of pulmonary tissue damage and lead to a faster recovery of lung function. Progesterone can promote the production of the epidermal growth factor amphiregulin which has also been shown to promote repair and recovery from influenza A virus infection. I hypothesized that the reparative effects of progesterone following influenza A virus infection would be mediated by upregulated amphiregulin, a critical protein for the proliferation of epithelial cells, to repair the damaged epithelium and promote recovery.

Specific Aim 2

My findings from Specific Aim 1 indicated that progesterone has a positive effect on primary influenza A virus infection by dampening inflammation and promoting repair. I wanted to further evaluate the effect of this anti-inflammatory environment on the induction of a memory humoral and cellular immune response. I hypothesized that by dampening inflammation, progesterone may hinder the generation of an optimal memory immune response. Few studies have evaluated the effects of progesterone on antibody production and those that have, show that progesterone decreases antibody responses. To date, no studies have assessed the effects of progesterone on memory CD8+ T cells responses. I hypothesized that progesterone would decrease total and neutralizing virus-specific antibodies and may impact protection following challenge with a secondary influenza A virus by lowering memory CD8+ T cells.

In this Specific Aim 2, after pilot studies showed that progesterone and related compounds worked equally well in ovariectomized and ovary-intact female mice, I elected to use ovary-intact mice to assess the effects of progesterone on influenza A virus infection and memory responses. For these studies, I also evaluated the effects of a synthetic progestin, levonorgestrel, on the outcome of influenza A virus infection. This progestin is widely used in hormonal-based therapies and binds to the progesterone receptor with a greater affinity than progesterone. I hypothesize that levonorgestrel, like progesterone, would dampen inflammation and promote protection from a primary influenza A virus infection with the caveat of decreasing antibody production and memory CD8+ T cell responses and protection against secondary infections.

Chapter 2

Progesterone-based Therapy Protects Against Influenza by Promoting Lung Repair and Recovery in Females

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Abstract

Over 100 million women use progesterone therapies worldwide. Despite having immunomodulatory and repair properties, their effects on the outcome of viral diseases outside of the reproductive tract have not been evaluated. Administration of exogenous progesterone (at concentrations that mimic the luteal phase) to progesterone-depleted adult female mice conferred protection from both lethal and sublethal influenza A virus (IAV) infection. Progesterone treatment altered the inflammatory environment of the lungs, but had no effects on viral load. Progesterone treatment promoted faster recovery by increasing TGF-β, IL-6, IL-22, numbers of regulatory Th17 cells expressing CD39, and cellular proliferation, reducing protein leakage into the airway, improving pulmonary function, and upregulating the epidermal growth factor amphiregulin (AREG) in the lungs. Administration of rAREG to progesterone-depleted females promoted pulmonary repair and improved the outcome of IAV infection. Progesterone-treatment of AREG-deficient females could not restore protection, indicating that progesterone-mediated induction of AREG caused repair in the lungs and accelerated recovery from IAV infection. Repair and production of AREG by damaged respiratory epithelial cell cultures in vitro was increased by progesterone. Our results illustrate that progesterone is a critical host factor mediating production of AREG by epithelial cells and pulmonary tissue repair following infection, which has important implications for women's health.

Author Summary

Worldwide, the use of hormonal contraceptives is on the rise as a primary intervention for improving women's health outcomes through reduced maternal mortality and increased childhood survival. There are many hormone contraceptive formulations, all of which contain some form of progesterone. Although the effects of hormone contraceptives and progesterone, specifically, have been evaluated in the context of infections of the reproductive tract, the effects of progesterone at other mucosal sites, including the respiratory tract have not been systematically evaluated. We have made the novel observation that administration of progesterone to female mice depleted of progesterone confers protection against both lethal and sublethal influenza A virus infection. In particular, progesterone reduces pulmonary inflammation, improves lung function, repairs the damaged lung epithelium, and promotes faster recovery following influenza A virus infection. Progesterone causes protection against severe outcome from influenza by inducing production of the epidermal growth factor, amphiregulin, by respiratory epithelial cells. This study provides insight into a novel mechanistic role of progesterone in the lungs and illustrates that sex hormone exposure, including through the use of hormonal contraceptives, has significant health effects beyond the reproductive tract.

Introduction

Hormonal contraceptives are listed as an essential medication by the World Health Organization (WHO)(289) because of the profound benefits these compounds can have on women's health outcomes, including decreased rates of maternal mortality and improved perinatal outcomes and child survival, by widening the intervals between pregnancies (290). Hormonal contraceptive formulations vary, but all contain some form of progesterone (P4) either alone or in combination with estrogen. There are currently over 100 million young adult women on P4-based contraceptives worldwide (4), with the WHO projecting that over 800 million women will be using contraceptives, including P4-based contraceptives, by 2030 (290). Despite the staggering numbers of women taking these compounds, very few studies evaluate the impact of contraceptives on responses to infection or vaccination, especially in non-sexually transmitted diseases.

Natural P4, produced by the ovaries during reproductive cycles, or synthetic P4 analogues found in contraceptives, signal primarily through progesterone receptors present on many cells in the body, including immune cells (e.g., NK cells, macrophages, dendritic cells (DCs), and T cells) as well as non-immune cells, such as epithelial cells, endothelial cells, and neuronal cells (12, 13). Human, animal, and *in vitro* studies show that P4 can alter the immune environment and promote homeostasis by decreasing inflammation and inducing anti-inflammatory responses. For example, in the presence of P4, macrophages and DCs have a lower state of activation, produce higher levels of anti-inflammatory cytokines, such as IL-10, and produce lower amounts of proinflammatory cytokines, such as IL-1β and TNF-α, as compared with placebo treated cells (33, 39). When either mice or cord blood cells from humans are treated with P4, the percentages of Foxp3+ regulatory T cells (Tregs) increase (57, 58). Although the immunomodulatory effects of P4-based therapies in the form of contraception have been studied in the context of sexually transmitted infections, including HIV and herpes simplex virus (92, 116, 291), the impact of P4

on the outcome of viral infectious diseases outside of the reproductive tract has not been considered in either humans or animal models.

Influenza A viruses (IAVs) primarily infect respiratory epithelial cells and induce the production of proinflammatory cytokines and chemokines that recruit immune cells, causing a local proinflammatory environment (149). Infiltration and activation of CD4+ and CD8+ T cells, while necessary for the clearance of IAVs (149, 292, 293), can trigger inflammation and lead to tissue damage and severe outcomes from IAV infection (267). Protection requires a balance between inflammatory responses generated to control virus replication and eliminate virus-infected cells with responses that mediate the repair of damaged areas of the lung. Repair involves a complex interplay among many cell types, cytokines, chemokines, growth factors, and extracellular matrix proteins that remodel tissue after acute injury, such as IAV infection (274). Amphiregulin (AREG) is an epidermal growth factor that has emerged as a significant mediator of tissue repair at mucosal sites, including the lungs (279, 280), gastrointestinal tract (282, 294), and reproductive tract (295, 296). Many immune cells produce AREG, but epithelial cells remain the principle producer of AREG following inflammation or tissue injury (273). If P4 can downregulate inflammatory immune responses and promote regulatory or tissue repair responses, then this hormone, at concentrations that reflect the luteal phase of the reproductive cycle, may improve the outcome of IAV infection.

Epidemiological and experimental evidence suggest that young adult females suffer a worse outcome than males following IAV infection, which in mice is associated with infection-induced suppression of reproductive hormones and excessive inflammatory immune responses in females (210, 222, 223). In addition to influenza, young adult females suffer a worse outcome than males from several autoimmune diseases, including multiple sclerosis (297). Paradoxically, a growing body of literature reveals that exogenous treatment of females (both humans and mice) with either estrogens or P4 limits inflammation and protects against infectious and autoimmune

diseases by decreasing inflammation and promoting repair (64, 222, 298, 299). In this series of studies, we show that treatment with sustained physiological doses of P4 protects females against IAV by reducing inflammation and improving pulmonary function, primarily through upregulation of AREG in epithelial cells. The observation that P4 regulates the cellular and molecular mediators of tissue repair at a mucosal site outside of the reproductive tract to restore tissue homeostasis after infection or injury has broad implications for women's health.

Results

Progesterone limits lung pathology and protects female mice against lethal IAV infection

To analyze the effects of P4 on morbidity and mortality in female mice, we depleted P4 by removing the ovaries and replaced P4 with subcutaneous pellets that delivered a continuous dose of physiological levels of P4 over the course of 21 days. Mice were subsequently mock-infected or infected with a dose of IAV (PR8) that is uniformly lethal for P4-depleted mice. Circulating levels of P4 and uterine horn mass, a biomarker of circulating P4 levels (300), were assessed over the course of infection to confirm the continuous effects of hormone replacement. Exogenous replacement of P4 significantly increased and sustained plasma P4 concentrations within the normal physiological range (301) throughout the duration of the study. Both mock- and IAV-infected females treated with exogenous P4 had higher circulating concentrations of P4, greater uterine horn mass, and higher expression of progesterone receptors (*Prs*) in the lungs than either mock or IAV-infected females treated with placebo throughout the 21 days (**Fig. 2.1 A and B**; $P<0.05$).

During the course of IAV infection, treatment of female mice with P4 mitigated the effects of infection on morbidity and mortality (**Fig. 2.1C and D**; $P<0.05$), with the average day of death being later for females treated with P4 (11.14±1.0 days post-infection [dpi]) as compared to placebo-treated females (9.5±0.6 dpi) ($P<0.05$). Progesterone treatment did not alter virus titers over the course of the first week of infection as compared to placebo treatment (**Fig. 2.1E**), suggesting that P4 did not render females more resistant to IAV infection. To test whether P4 improved survival during IAV infection by making females more tolerant to the negative consequences of infection on host health, we analyzed the interaction between virus titers and body temperature during peak disease (7dpi) (302). Females treated with P4 suffered less hypothermia relative to their pulmonary viral load than the placebo-treated females, suggesting that P4 made females more tolerant of IAV infection (**Fig. 2.1F**; $P<0.05$). To test the hypothesis

that P4 may increase tolerance by reducing inflammation and damage in the lung, pulmonary tissue was evaluated for vasculitis, bronchiolitis, alveolitis, and edema. In mock-infected animals, P4 alone did not result in changes in any of the parameters examined (**Fig. 2.1G** [panels 1 and 2]). Seven days post-infection with IAV, treatment with P4 decreased vasculitis (**Fig. 2.1G** [panels 3 and 4] and H) and edema (**Fig 2.1G** [panels 5 and 6] and H) as compared to the placebo-treated mice ($P<0.05$). Progesterone improved the outcome of lethal IAV infection by limiting lung inflammation and damage, but not virus replication.

Virus-specific CD8+ T cells are necessary for clearance of IAV but can also contribute to immunopathology (197, 303). Although the total numbers of CD8+ T cells increased in all females following IAV infection, the total number of CD8+ T cells, the number of IAV-specific CD8+ T cells, and the production of IFN-γ and TNF-α by virus-specific CD8+ T cells in the lungs did not differ between P4- and placebo-treated females (**Table 2.1**). These data indicate that P4 did not affect the cell-mediated antiviral immune response during acute IAV infection.

Progesterone promotes a repair environment in the lungs during lethal IAV infection

IAV infection is characterized by the induction of a cytokine storm and excessive immunopathology, which leads to tissue damage (265). Damage to the lung endothelium and/or epithelium results in vascular leakage into the air spaces, and can be quantified by measuring protein concentration in bronchoalveolar lavage (BAL) fluid. Consistent with the histopathological findings of increased vasculitis and edema (**Fig. 2.1H**) following lethal IAV infection, treatment of females with P4 decreased the total amount of protein contained in the BAL as compared to placebo-treated mice (**Fig. 2.2A**; $P<0.05$). Among infected females, treatment with P4 also increased cellular proliferation (as measured by Ki67 expression) in the lungs as compared to placebo treatment during peak disease (7dpi) (**Fig. 2.2B and C**; $P<0.05$). Analysis of the expression of Ki67 in the different areas of the lungs revealed greater proliferation in several regions of the lungs, but was most pronounced in the epithelial cells lining

the airways during IAV infection in P4-treated mice (**Fig. 2.2C**). The epidermal growth factor, AREG, promotes proliferation of epithelial cells and protects mice from excessive pathology during IAV infection (279, 280). Analysis of AREG expression during peak disease (7 dpi) revealed increased mRNA expression, as well as AREG protein in the bronchioles but not the alveoli, in the lungs of P4-treated mice as compared to placebo-treated mice infected with IAV (**Fig. 2.2D-F**, $P<0.05$).

Progesterone induces Th17 cells in the lungs of IAV-infected female mice

Progesterone treatment altered inflammation during IAV infection (**Fig. 2.1G and H**) and induced a repair environment through cellular proliferation and restoration of barrier integrity (**Fig. 2.2A-C**). To further characterize the effect of P4 on inflammatory responses to IAV, a panel of 13 cytokines and chemokines was analyzed in the supernatant of whole lung homogenates. As expected, following infection with IAV, pulmonary concentrations of IL-1β, TNF-α, IFN-γ, and IL-12p70 were significantly increased during the first week of infection in all females, regardless of P4 treatment (**Suppl. Table 2.1**; $P<0.05$). P4 treatment decreased pulmonary production of the alarmins IL-13 and IL-33 as compared with placebo treatment during IAV infection (**Suppl. Table 2.1**; $P<0.05$). The only two cytokines that were significantly increased in P4-treated females compared with placebo-treated females during IAV infection were IL-6 and TGF-β (**Fig. 2.3A and B**; $P<0.05$). P4 treatment of IAV-infected mice had no effect on the other canonical regulatory protein, IL-10, as compared to placebo treatment (**Suppl. Table 2.1**).

Production of TGF-β and IL-6 increases differentiation of Th17 cells. Th17 cells promote repair of the gut epithelium (304) and may be similarly involved in orchestrating repair of the pulmonary epithelium. To test this hypothesis, populations of CD4+ T cells from mock- and IAV-infected mice were enumerated during peak disease (7 dpi). There was no effect of P4 treatment on total numbers of CD4+ T cells, Th1, Th2, or Treg cells in the lungs at 7 dpi (**Table**

2.1). In contrast, P4 treatment increased the total number of Th17 cells in the lungs during IAV infection as compared with placebo treatment (**Fig. 2.3C**; $P<0.05$). The cytokine IL-23 is necessary for maintenance of Th17 cells and the expression of *Il23* mRNA in the lungs was increased in P4- compared with placebo-treated females (**Fig. 2.3D**; $P<0.05$). Th17 cells exert their tissue reparative effects by increasing the production of IL-22 (305). The expression of *Il22* mRNA in the lungs was greater in P4- than placebo-treated females during IAV infection (**Fig. 2.3E**; $P<0.05$). Finally, one surface marker on Th17 cells that is associated with reducing inflammation (i.e., regulatory or suppressive Th17 cells) is the ectonucleotidases CD39 (306, 307). The percentage of Th17 cells that expressed CD39 was significantly increased in P4-treated as compared to placebo-treated females during IAV infection (**Fig. 2.3F**; $P<0.05$). These data indicate that P4 alters the inflammatory milieu of the lungs by promoting a repair environment in IAV-infected female mice, with increased numbers of regulatory Th17 cells, elevated expression of *Il22*, and upregulated expression of *Areg* during lethal IAV infection.

Progesterone accelerates long-term pulmonary recovery during sublethal IAV infection

To further evaluate the role of P4 in lung repair and recovery from IAV infection, P4- and placebo-treated female mice were infected with a less pathogenic IAV strain, ma2009, at a dose ($0.4mLD_{50}$) that allowed for monitoring of the mice over a longer duration of time. Similar to lethal IAV infection, P4-treated females infected with sublethal IAV showed less hypothermia (**Fig. 2.4A**; $P<0.05$) and reduced clinical disease (**Fig. 2.4B**; $P<0.05$) as compared to placebo-treated females. Analysis of pulmonary virus titers confirmed that P4 did not alter virus titers or clearance of infectious virus over the course of IAV infection (**Fig. 2.4C**). To determine if P4 reduced cell death due to IAV infection, LDH levels in the BAL fluid were quantified. Cellular damage during IAV infection was not altered by treatment with P4 as compared with placebo (**Fig. 2.4D**). Lung sections were evaluated for markers of inflammation and damage during the recovery (14 dpi) and post-recovery (25 dpi) phases of IAV infection. At 14 dpi, but not at 25 dpi,

treatment of IAV-infected female mice with P4 decreased the percentage of lesioned areas, alveolitis, edema, and cumulative inflammation as compared to placebo-treated mice (**Fig. 2.4E-H**, $P<0.05$). Treatment with P4 significantly increased Ki67 expression in pulmonary cells during the recovery phase (14 dpi) of IAV infection as compared with placebo treatment (**Fig. 2.4I**; $P<0.05$). Based on the observation that P4 treatment promoted lung repair in IAV-infected female mice, we evaluated the impact of P4 on overall lung physiology during (14 dpi) and after (25 dpi) recovery from sublethal IAV infection by assessing markers of pulmonary function. Lung diffusing capacity (DF_{CO}), lung tissue compliance (Crs), and resistance (Rrs) returned to baseline faster in P4- than placebo-treated mice infected with IAV (**Fig. 2.4J-L**, $P<0.05$). Treatment of female mice with P4 reduces inflammation and promotes faster recovery from sublethal IAV infection.

The protective effects of P4 against influenza are mediated by AREG

Progesterone increased pulmonary AREG expression during lethal IAV infection (**Fig. 2.2D-F**) and increased AREG expression is associated with an improved outcome from lethal IAV infection (279, 280). In our sublethal IAV model, we were able to measure pulmonary expression and production of AREG over a longer duration of time to establish the effects of P4 on the kinetics of AREG production in females. P4-treatment induced a 30-70 fold greater induction of *Areg* mRNA and higher concentrations of AREG protein in the lungs as compared with placebo treatment over the course of IAV infection (**Fig. 2.5A and B**; $P<0.05$). Peak production of AREG occurred at 9 dpi (**Fig. 2.5B**), which corresponded with peak disease (**Fig. 2.4A and B**) during sublethal IAV infection. To test the hypothesis that reduced AREG production in P4-depleted females caused a more severe outcome from IAV, we treated P4-depleted female mice with recombinant AREG (rAREG) during the course of IAV infection. Treatment of P4-depleted mice with rAREG resulted in AREG levels that were comparable to those of P4-treated mice at 14 dpi (**Fig. 2.5C**; $P<0.05$). Treatment of P4-depleted females with

rAREG significantly improved the recovery from IAV infection (**Fig. 2.5D and E**; $P<0.05$), with reduced inflammation (**Fig. 2.5F and G**; $P<0.05$) and improved pulmonary function, including lung diffusing capacity (DF_{CO}), lung compliance (Crs), and resistance (Rrs), to levels similar to that of P4-treated females (**Fig. 2.5H-J**; $P<0.05$). These data suggest that the protective effects of P4 on IAV disease may be mediated by an upregulation of AREG.

The contribution of AREG to P4-mediated protection from IAV infection was further determined by using mice that lacked the expression of a functional *Areg* gene (308). Deletion of the *Areg* gene in female mice ($Areg^{-/-}$) reversed the protective effects of P4 on the outcome of IAV infection (**Fig. 2.6A and B**; $P<0.05$). This was accompanied by increased inflammation in P4-treated *Areg-/-* as compared with WT female mice (**Fig. 2.6C and D**; $P<0.05$). Improvement of pulmonary function in the presence of P4, as measured by lung diffusing capacity (DF_{CO}), compliance (Crs), and resistance (Rrs), was also reversed in IAV-infected $Areg^{-/-}$ mice as compared with WT mice treated with P4 (**Fig. 2.6E-G**; $P<0.05$). Taken together, these data indicate that P4 treatment of IAV-infected female mice promotes a pulmonary repair environment and restoration of lung function through the induction of AREG.

Progesterone accelerates wound healing and increases production of AREG by respiratory epithelial cells

Treatment with P4 induces higher expression of AREG in the lungs of sublethal IAV-infected females, particularly in the epithelial cells lining the larger airways, as compared with placebo-treatment (**Fig. 2.7A and B**; $P<0.05$). To assess the contribution of P4 treatment to the repair of damaged respiratory epithelia, we used an *in vitro* model system in which primary, differentiated mouse tracheal epithelial cell (mTECs) cultures were mechanically injured. The mTECs express the progesterone receptor (*Pr*), which was upregulated in the presence of P4 (**Fig. 2.7C**; $P<0.05$). Repair of the epithelial cell layer was measured over time to identify the return of the transepithelial resistance (TER) to baseline. Following injury, cultures of mTECs treated with

P4 returned to baseline TER faster than vehicle-treated cultures (**Fig. 2.7D**; $P<0.05$). During injury, mTEC cultures treated with P4 produced more AREG mRNA and protein than vehicle-treated mTECs cultures (**Fig. 2.7E and F**; $P<0.05$). These data illustrate that P4 improves pulmonary repair and function by increasing AREG production and wound repair in epithelial cells.

Discussion

Hosts have evolved several mechanisms for overcoming viral infections, such as the induction of antiviral defenses that increase resistance to infection, or the activation of regulatory and repair responses that increase tolerance to the negative consequences of infection. In the present study, P4 significantly protected females during IAV infection by altering inflammation, improving pulmonary function, and promoting a pulmonary repair environment, which resulted in an earlier recovery. The protective effects of P4 were primarily mediated by the induction of AREG during both lethal and sublethal infections. Progesterone did not increase resistance to infection in females as demonstrated by the lack of an effect of P4 treatment on virus titers, clearance of infectious virus, numbers of Th1 cells, and CD8+ T cell activity in lungs. Instead, P4 reduced the detrimental consequences of IAV infection in females by increasing their tolerance to infection. Several host immunological factors, including TGF-β, Tregs, and regulatory populations of CD39+ Th17 cells, are associated with maintaining the balance between protective and pathological immune responses during IAV infection. Although P4 treatment had no effect on the numbers of Tregs in the lungs during IAV infection, concentrations of TGF-β and IL-6, the expression of *Il23* and *Il22*, the number of Th17 cells, as well as the proportion of Th17 cells expressing CD39, were increased. Regulatory Th17 cells express the ectonucleotidases CD39 and are associated with repair following inflammation and infection (306, 307). Th17 cells also promote epithelial cell proliferation and repair in the gut, primarily through the induction of IL-22 (304). Consequently, treatment of females with P4 increased IL-22, a cytokine that has been

shown to mediate regeneration of lung epithelial cells following IAV infection (277). Whether the P4-induced increase in regulatory Th17 cells and IL-22 promotes cellular proliferation and repair of the lung epithelium during IAV infection by increasing AREG production requires consideration. Because P4 directly induced AREG production in respiratory epithelial cells *in vitro*, P4-induced AREG production may occur independent of the reparative effects of regulatory Th17 cells in the lungs during IAV infection.

Progesterone induces repair of epithelial cells in the endometrium and myelin fibers in the central nervous system (32, 309). This repair of myelin fibers by P4 (17) is one factor mediating how this reproductive hormone mitigates the progression of multiple sclerosis (64). Our data show that P4 promotes proliferation of pulmonary cells, including epithelial cells, and pulmonary tissue repair. The reparative effects of P4 in the reproductive tract are caused by the induction of AREG, which promotes epithelial remodeling in mammary and uterine tissues (295, 296). In the respiratory tract, AREG is involved in pulmonary tissue remodeling and repair during lung injury, asthma, and infection (279, 280, 282-284). Although *Areg*-gene deficient mice show few abnormalities under homeostatic conditions (308), their ability to resolve inflammation or infection is severely impaired (282, 294). During IAV infection, administration of rAREG protects mice from severe IAV-mediated morbidity by decreasing hypothermia, improving pulmonary function, and decreasing protein leakage into the airways (279, 280). The data presented are the first report of P4 induction of AREG outside of the reproductive tract and in the context of infection. The effect of other reproductive hormones on AREG expression, including differential expression between males and females, warrants further study.

AREG is produced primarily by epithelial cells (287), but type 2 innate lymphoid cells (ILC2) and Tregs have also been shown to produce AREG during IAV infection and contribute to the repair during resolution of infection (279, 280, 287, 288). Because each of these cell type express progesterone receptors (13, 310), each is a potential producer of AREG in response to P4

treatment. Our *in vivo* and *in vitro* data suggest that respiratory epithelial cells are a predominant source of P4-induced AREG. Following IAV infection, AREG expression was predominantly localized to the bronchiolar epithelial cells, and P4 treatment of isolated mTECs increased AREG production following mechanical damage. Furthermore, P4-treatment did not activate markers of ILC2s, including IL-13 and IL-33 production, or increase numbers of Tregs in the lungs during infection, suggesting that the induction of AREG in response to P4 may not be occurring in these immune cell populations.

Recovery following IAV infection is generally defined as a return of body temperature or body mass back to homeostatic levels (311). In this study, however, we showed that pulmonary pathology and impaired pulmonary function persisted after measures of overall health, including hypothermia and clinical disease, returned to baseline. Furthermore, the impact of IAV infection was observed long after infectious virus had been cleared from the lungs. Recovery following IAV infection extended beyond 21 dpi and should be defined not only by reduced morbidity, but also by restored pulmonary function, both of which were expedited by P4 treatment in females.

Progesterone concentrations fluctuate naturally during the female life span, with moderate concentrations during the menstrual cycle, high concentrations during pregnancy, and low concentrations following menopause. Progesterone is also used exogenously by over 100 million women worldwide in P4-based hormonal contraceptives, by post-menopausal women taking hormonal replacement therapy, and by both men and women in the treatment of cancer, osteoporosis, and brain injury (4, 8). Prior to this study, the health consequences of P4-based therapies in acute respiratory infection had not been characterized. We have demonstrated that AREG, which is a significant factor that induces tissue repair and recovery from infectious diseases, is regulated by P4 during both lethal and sublethal IAV infection. The data presented provide critical mechanistic information about how P4 and possibly synthetic P4 analogues affect women's health outside of the reproductive tract. Contraceptives that contain P4 are listed as an

essential medication by the WHO, being a safe and effective method for improving health outcomes in women, including those living with HIV (289). During outbreaks of infectious diseases that harm pregnant women and their fetuses (e.g., the current Zika outbreak), the WHO recommends increased use of hormonal contraceptives, which according to our data could have additional beneficial consequences on the outcome of other infectious diseases.

Material and Methods

Ethics statement

All experiments were performed in compliance with the standards outlined in the National Research Council's Guide to the Care and Use of Laboratory Animals. The animal protocol (M015H236) was reviewed and approved by the Johns Hopkins University Animal Care and Use Committee. All efforts were made to minimize animal suffering.

Animals

Adult (7–8 weeks old) female C57BL/6 mice were purchased from NCI Frederick. $Areg^{+/-}$ (C57BL/6 129 Sv) mice were kindly provided by Dr. Marco Conti (University of California San Francisco) and bred to obtain $Areg^{-/-}$ and $Areg^{+/+}$ female littermates. Mice were housed 5 per microisolator cages under standard BSL-2 housing condition with food and water ad libitum.

Surgical procedures

At 8–12 weeks of age, mice were anesthetized with an intramuscular injection of ketamine (80 mg/kg) and xylazine (8 mg/kg) cocktail and ovaries were removed bilaterally as previously described (222). All animals were given two weeks to recover prior to infection. Recombinant amphiregulin (10μg; R&D) was administered intraperitoneally every other day using saline as the vehicle.

Hormone replacement and quantification

Ovariectomized (ovx) mice were assigned to receive subcutaneous implants of placebo (-P4) or 15 mg progesterone (+P4) 21-day release pellets (Innovative Research of America) prior to IAV inoculation. Circulating concentrations of P4 were assessed from plasma using ether extraction and radiolabelled immunoassay, with P4 antibody (MP Biomedicals) and tracer 3H-P4 (American Radiolabeled). Uterine horns were removed at several time-points upon euthanasia of mice and wet weight was quantified as a bioassay for P4.

Virus infection and quantification

Mouse-adapted influenza A viruses, A/Puerto Rico/8/34 (PR8; H1N1) provided by Dr. Maryna Eichelberger at the Food and Drug Administration (FDA) and A/California/04/09 (ma2009; H1N1) generated by Dr. Andrew Pekosz from a published sequence (251), were used in these studies. Mice were anesthetized and inoculated intranasally with 30 μl of DMEM (mock) or H1N1 virus (1.78 50% mouse lethal dose (MLD_{50}) for PR8 and 0.4 MLD_{50} for ma2009). Clinical disease scores for IAV-infected mice were based on four parameters, with one point given for each of the following: dyspnea, piloerection, hunched posture and absence of an escape response. For virus quantification, $\log_{10}$ dilutions of lung homogenates (starting at 10^{-1}) were plated onto a monolayer of MDCK cells in replicates of 6 for 4–6 days. Cells were stained with naphthol blue black (Sigma Aldrich) and scored for cytopathic effects. The 50% tissue culture infectious dose ($TCID_{50}$) was calculated according to the Reed-Muench method.

Cytokine and chemokine quantification

Snap-frozen lung tissue was homogenized in DMEM supplemented with 1% penicillin/streptomycin and 1% L-glutamine (Invitrogen) and centrifuged to remove cellular debris. Supernatants were harvested to measure IL-1β, TGF-β, IL-4, IL-5, IL-13, IL-17, IL-33, and AREG by ELISA (R&D Systems and BD Biosciences) and CCL-2, IL-12(p70), TNF-α, IFN-γ, IL-6 and IL-10 with the mouse inflammation cytometric bead array (BD Biosciences) according to the manufacturer's protocols.

Real time reverse transcription PCR

Snap-frozen lung tissue or mTECs were homogenized in TRIzol and RNA was purified by chloroform extraction. RNA concentration and purity was measured using a NanoDrop (ThermoFisher Scientific). The RNA concentration in each sample was standardized to 1 μg using RNAse-free water. Reverse transcription was carried out using the iScript cDNA synthesis

kit (Biorad) according to the manufacturer's protocol. Pre-designed Il23 (Mm.PT.58.10594618.g), Il22 (NM_016971.2), Areg (Mm.PT58.31037760), Gapdh (Mm.PT.39a.1) and Pr (Mm. PT.58.10254276) PrimeTime Primers were purchased from Integrated DNA Technologies. Semi-quantitative RT-PCR was performed in 96-well optical reaction plates using the SsoFast EvaGreen Supermix (Biorad) on the StepOnePlus RT-PCR system (Applied Biosystems). Gene expression was normalized to Gapdh and mock-infected samples or wells with no injury using the ΔΔCt method.

Flow cytometry analyses of T cells

Lungs were excised and single-cell suspensions were generated following red blood cell lysis. Total viable cells were determined using a hemocytometer and trypan blue (Invitrogen) exclusion and resuspended at $1x10^6$ cells/ml in RPMI 1640 (Cellgro) supplemented with 10% FBS (Fisher Scientific) and 1% penicillin/streptomycin. For IAV-specific T cells enumeration, cells were cultured for 5h with IAV peptide antigen (CD8:$NP_{366-374}$, or CD4: $HA_{211-255}$, $NP_{311-325}$, respectively) (ProImmune) in media containing Brefeldin A (GolgiPlug, BD) The viability of cells was determined by fixable Live/Dead violet viability dye (Invitrogen) and Fc receptors were blocked using anti-CD16/32[A]. The T cell populations were stained with the following antibodies: PerCP-Cy5.5 conjugated anti-CD4 (RM4-5)[A], PerCP-Cy5.5 conjugated anti-CD8 (53–6.7)[A], FITC conjugated anti-CD25 (7D4)[A], PE conjugated $D^bNP_{366-374}$ tetramer (NIH Tetramer Core Facility), FITC conjugated anti-CD4 (RM4-5)[B], APC conjugated anti-CD3 (17A2[B], and PerCP-eFluor 710 conjugated anti-CD39 (24DMS1)[B]. Intracellular staining with PE conjugated anti-TNF-α(MP6-XT22)[A], FITC conjugated anti-IFN-γ (XMG1.2)[A], PE conjugated anti-IL-4 (11B11)[A], and PE conjugated anti-IL-17 (TC11-1810)[A], was performed following permeabilization and fixation with Cytofix/Cytoperm and Perm/Wash buffer[A]. Intracellular staining with PE-conjugated Foxp3 (MF23)[A] was performed following fixation and permeabilization with a Foxp3 staining buffer set[A]. Data were acquired using a FACS Cali- bur

(Cellquest Software) and analyzed using FlowJo (Tree Star, Inc.). Total cell counts were determined by multiplying each live cell population percentage by the total live cell counts acquired prior to staining by trypan blue exclusion counts on a hemocytometer. All reagents were purchased from BD Biosciences[A] or eBioscience[B] unless stated otherwise.

Histopathology and immunohistochemistry

Lungs were inflated, fixed in Z-fix (Anatech), embedded in paraffin, cut into 5μm sections, and mounted on glass slides. Slides were stained with hematoxylin and eosin (H&E) and used to evaluate lung inflammation. Histopathological scoring was performed by a single blinded veterinary pathologist on a scale from 0–3 (0, no inflammation; 1, mild inflammation; 2, moderate inflammation; and 3, severe inflammation) for the following parameters: bronchiolitis, alveolitis, vasculitis, perivasculitis, necrosis, consolidation, and edema (155, 312). The sum of these parameters represents the cumulative inflammation score. The percentage of lesioned areas within each tissue section was also evaluated. Histopathological slides were deparafinized with xylene and rehydrated in graded ethanol. Heat-induced antigen retrieval with citrate buffer was performed and slides were blocked with 10% normal serum prior to overnight primary antibody incubation. For Ki67, rabbit anti-Ki67 (1/200; Abcam) was used, detected with the EXPOSE rabbit specific HRP/DAB detection kit (Abcam), counterstained with Hematoxylin and slides were mounted using Permount (Fisher). For immunofluorescence, anti-AREG (1/ 100; R&D) and anti-β-tubulin IV (1/100; BioGenex) were used and detected with appropriate secondary antibodies (1/400) conjugated to AF-555 (Thermo) and AF488 (Molecular probes). Slides were then treated against autofluorescence using 0.3% Sudan Black B (Sigma) in 70% ethanol and mounted using anti-fade medium containing DAPI (ProLong Gold from Cell Signaling Techonology). Images were taken using a Nikon Eclipse E800 (for H&E and Ki67 stains) or a Zeiss AxioImager M2 (for immunofluorescence) and analyzed using ImageJ (NIH).

Bronchoalveolar lavage

Mice were euthanized by cervical dislocation and the lungs were lavaged twice with 0.5ml of a 0.9% saline solution. Bronchoalveolar lavage (BAL) fluid was centrifuged at 500g for 10 minutes to remove cells and debris and the supernatant was collected to quantify total protein leak- age into the airway using a BCA assay (Pierce). Cell lysis and damage was analyzed from BAL fluid by measuring lactate dehydrogenase leakage using an LDH assay kit (Sigma).

Pulmonary function phenotyping

Lung Diffusing Capacity (DF_{CO}) quantifies the ability of the lung to exchange gas, which is its primary function. Diffusing capacity is simple and quick to measure in humans and mice, and it decreases with nearly all lung pathologies, including viral infections. At the selected time points, a cohort of mice was anesthetized via an IP injection of ketamine–xylazine (100 mg/ kg–10 mg/kg), and then an 18-g stub needle was secured in the trachea. 0.8 mL of a gas mixture containing 0.3% neon, 0.3% CO in room air was quickly injected into the lungs, held for 9 s, then quickly withdrawn. This post breathold sample was then injected into a desktop gas chromatograph (Inficon, Micro GC model 3000A) to measure the concentrations of Ne and CO. The DF_{CO} in mice is analogous to the DL_{CO} in humans, and is calculated as $1-(CO9/COc)/(Ne_9/Ne_c)$, where subscripts c and 9 refer to the calibration gas injected and the gas from the 9 s exhaled sample. DF_{CO} is thus a dimensionless variable which varies between 0 and 1, and is used to detect the loss and recovery of lung function after the viral infections used in this study (313).

Lung mechanics: After the DF_{CO} is measured, the tracheostomy cannula was then connected to a Flexivent system (Scireq). Ventilation was accomplished at a rate of 150 breaths/ minute and a tidal volume of 10 ml/kg with a PEEP of 3 cm H_2O. A deep inspiration to 30 cmH_2O was done, and 1 minute later the respiratory resistance (Rrs) and compliance (Crs) were measured (314).

Increased resistance reflects increased difficulty in dynamically moving air into the lung and decreased compliance reflects increased difficulty in expanding the lung parenchyma.

Mouse tracheal epithelial cell (mTEC) cultures

For mTEC cultures, tracheas were obtained from 7–9 week old C56BL/6 female mice, digested overnight in 0.3% pronase, and enriched by depleting fibroblasts as previously described (315, 316). The mTECs were cultured at a density of $2.22x10^5$ cells/ml on collagen-coated 24-well transwell plates for 7 days (i.e., until the cultures reached a transepithelial resistance above 1000 $\Omega \cdot cm^2$) and apical medium was removed to create an air-liquid interface for 14 days to induce differentiation as described previously (316). Cells were pre-treated for 24 h with basolateral media containing vehicle (100% ethanol) or 100nM P4 (Sigma), and injured by scratching the cell layer with a 10ul XL pipette tip, or left uninjured, and loose cells were removed by washing with media. Transepithelial cell resistance (TER) was measured prior to injury, immediately after, and every 12h for 48 h by adding 100µl of warm TEC basic media to the apical chamber. New media with vehicle or P4 was added every 24h. Every 12h, basolateral media was sampled and analyzed for AREG expression by ELISA (R&D) according to the manufacturer's protocol. Cells were harvested in Trizol every 12h and analyzed by RT-PCR as described above.

Statistical analyses

A power and sample size calculation was used to confirm group sizes for a power of 0.8 and contributes to differential sample sizes for some dependent measures. Repeat measures were analyzed with a multivariate analysis of variance (MANOVA) followed by planned comparisons. Discrete measures were analyzed with T-tests or two-way ANOVA followed by the Tukey method for pairwise multiple comparisons. Survival was analyzed using a Kaplan Meyer survival curve followed by a log-rank test. Mean differences were considered statistically significant if $P<0.05$.

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Figure and table legends

Figure 2.1. Progesterone (P4) protects adult female mice against lethal IAV infection. Adult female mice were ovariectomized, treated with placebo (-P4) or exogenous P4 (+P4), and inoculated with lethal IAV or mock-infected. Serum was collected at 3, 5, 7, and 21 days post-inoculation (dpi) and P4 concentrations (mock n = 5, IAV n = 20–22 [i.e., n = 5–7 per dpi]) were analyzed by radioimmunoassay, and uterine horns (mock n = 13, IAV = 35–38 i.e., n = 12–14 dpi time-point) were weighed (A). Lungs were harvested at days 3, 5, or 7 dpi and mRNA expression of the progesterone receptor (Pr) was measured and normalized to GAPDH and mock-infected animals using the ΔΔCt method (B). Values for each measure (A and B) did not differ between dpi and are shown as aggregates. Mice (-P4 n = 20, +P4 n = 10) were monitored daily for changes in rectal body temperature (C) and survival (D) for 21 dpi. Infectious virus titers in the lungs were measured at 3, 5, or 7 dpi (E; n = 8-10/treatment/dpi). The correlation between changes in body temperature and virus titers at 7dpi, as a measure of disease tolerance, was quantified using a linear regression model (F; n = 12/treatment). H&E stained lung sections collected at 7dpi from mock-infected (G, panel 1 and 2) and IAV-infected females (G, panels 3–6) were scored for inflammation. Alveolitis (G panel 3 and 4, indicated by black triangles) and edema are shown (G panels 5 and 6, indicated by black stars), as well as corresponding histopathogical scores on a scale from 0–3 (H, n = 3/treatment, 10 fields/animal, 10X magnification). Data represent means ± SEM from two independent experiments and significant differences are represented by asterisks (*).

Figure 2.2. Progesterone (P4) treatment promotes barrier integrity, cellular proliferation, and induction of amphiregulin (AREG) in the lungs of IAV- infected female mice. Adult female mice were ovariectomized, treated with placebo (-P4) or exogenous P4 (+P4), and inoculated with lethal IAV or mock-infected. At peak disease (7dpi), bronchoalveolar lavage (BAL) fluid or whole lungs were harvested or fixed for histology. Total protein content in the

BAL was measured by BCA assay (A). Cellular proliferation was assessed at 7 dpi using the marker Ki67 in paraffin-embedded lung tissue sections; sections were counterstained with hematoxylin; and arrows indicate examples of Ki67+ cells (C). The percentages of Ki67+ cells were analyzed and quantified using ImageJ (B and C; 10X magnification). Areg mRNA expression was quantified and normalized to Gapdh and to mock-infected controls (D). The percentages of AREG+ cells (red) in bronchioles and alveolar airspace were analyzed using immunofluorescence and quantified using ImageJ (E; n = 20 fields/treatment). Representative images of bronchioles (10X magnification) and focused areas (40X magnification) with epithelial cells (β- tubulin^{+} cells, in green) are shown (F). Bars represent means ±SEM from two or three independent experiments. Significant differences are represented by an asterisk (*) (mock: n = 6; IAV: n = 10–12).

Figure 2.3. Progesterone (P4) treatment induces regulatory Th17 cells in the lungs of IAV-infected female mice. Adult female mice were ovariectomized, treated with placebo (-P4) or exogenous P4 (+P4), and inoculated with lethal IAV or mock-infected. At peak disease (7 dpi), supernatant from whole lung homogenates was used to quantify IL-6 (A) and TGF-β (B). The total numbers of Th17 (C) cells were measured by flow cytometry in lung single-cell suspensions stimulated ex vivo with IAV-specific antigen ($D^{b}HA_{211-255}$ and $D^{b}NP_{311-325}$) in presence of BFA. The expression of Il23 and Il22 was analyzed in lung tissue and normalized to GAPDH and to mock-infected controls using the ΔΔCt method (D and E). Expression of CD39 was evaluated by flow cytometry on Th17 cells (F) from lung single-cell suspensions. Bars represent means ±SEM from two or three independent experiments. Significant differences are represented by an asterisk (*) (mock: n = 6; IAV: n = 10–12).

Figure 2.4. Progesterone (P4) reduces inflammation and improves pulmonary function during sublethal IAV infection. Adult female mice were ovariectomized, treated with placebo (-

P4) or exogenous P4 (+P4), and inoculated with a sublethal dose of IAV or mock-infected. Females (n = 23-25/treatment) were monitored daily for changes in rectal body temperature (A) and clinical disease (B) for 21 dpi. Infectious virus titers (C) and cell necrosis (D) were measured 3, 5, 7, 9 and 14 dpi (n = 5–10 per dpi). Percentage of lesioned areas (E), alveolitis scores (F), edema scores (G), and cumulative inflammation scores (H) were quantified in H&E stained lung sections at 14 and 25 dpi. The numbers of proliferating of Ki67+ cells were analyzed at 14 and 25 dpi and quantified using ImageJ (I) (n = 3-5/treatment/dpi with 10 fields per animal). Pulmonary function tests, measuring lung diffusing capacity (DF_{CO}; J), lung tissue compliance (Crs; K), and resistance (Rrs; L), were performed at 14 and 25 dpi with the dotted line representing the average value (mean ±SEM) for mock-infected mice (n = 7-10/treatment/dpi). Data represent means ±SEM from 2–3 independent experiments and significant differences are represented by asterisks (*).

Figure 2.5. Progesterone (P4) increases amphiregulin (AREG) expression and administration of recombinant AREG protects P4-depleted female mice against IAV infection. Adult female mice were ovariectomized, treated with placebo (-P4) or exogenous P4 (+P4), and inoculated with a sublethal dose of IAV or mock-infected. The expression of amphiregulin (Areg) mRNA (A) and protein concentrations (B) in the lungs were quantified at 3, 5, 7, 9 and 14 dpi (n = 8-10/treatment/dpi). Gene expression was normalized to Gapdh and mock-infected controls using the ΔΔCt method. Ovariectomized mice were treated with placebo (-P4), placebo and recombinant amphiregulin (-P4 +rAREG), or P4 (+P4) and inoculated with a sublethal dose of IAV. To confirm AREG replacement, pulmonary concentrations of AREG were measured at 14 dpi (C). Mice were monitored daily for changes in body temperature (D) and clinical disease (E) (n = 9-10/treatment). H&E stained lung sections collected at 14 dpi were scored for inflammation as a cumulative score of perivasculitis, vasculitis, bronchiolitis, alveolitis, edema, consolidation, and necrosis (F). Representative images of overall inflammation

(2X magnification) and focused areas (10X magnification) with cellular infiltration and edema are shown (G) (n = 3-5/treatment, with 10 fields per animal). Pulmonary function tests were performed at 14 dpi and lung diffusing capacity (DF_{CO}; H), lung compliance (Crs; I), and resistance (Rrs; J) were measured (n = 8-10/treatment). The dotted lines represent the value (means ±SEM) for mock-infected mice and bars and circles represent means ±SEM for IAV-infected mice from 2 independent experiments, with significant differences represented by asterisks (*).

Figure 2.6. Deletion of amphiregulin (Areg) reverses the protective effects of progesterone (P4) during IAV infection. Female $Areg^{-/-}$ or WT littermates were ovariectomized, treated with P4, inoculated with a sublethal dose of IAV or mock-infected, and monitored daily for changes in body temperature (A) and clinical disease (B) (n = 15/treatment). At 14 dpi, inflammation was scored from H&E stained lung sections for inflammation as a cumulative score of perivasculitis, vasculitis, bronchiolitis, alveolitis, edema, consolidation and necrosis (C). Representative images of overall inflammation (2X magnification) and focused areas (10X magnification) with cellular infiltration and edema are shown (D) (n = 5/treatment, with 10 fields per animal). Pulmonary function tests were performed at 14 dpi and lung diffusing capacity (DF_{CO}; E), lung compliance (Crs; F), and resistance (Rrs; G) were measured (n = 10-12/ treatment). The dotted lines represent the value (means ±SEM) for mock-infected Areg-/- mice and bars and circles represent means ±SEM for IAV- infected mice from 2 independent experiments, with significant differences represented by asterisks (*).

Figure 2.7. Progesterone induces amphiregulin (AREG) and accelerates wound healing in respiratory epithelial cells. Adult female mice were ovariectomized, treated with placebo (-P4) or exogenous P4 (+P4), and inoculated with a sublethal dose of IAV. Animals were euthanized at 14 dpi and lungs were fixed for histology (n = 5/treatment). AREG expression (in red) was

assessed in epithelial cells (β-tubulin^{+} cells, in green) using immunofluorescence in different areas of the lung tissue (A). Representative images of bronchioles (10X magnification) and focused areas (40X magnification) are shown. The percentages of AREG+ cells in bronchioles and alveolar airspace were analyzed and quantified using ImageJ (B; n = 20 fields/treatment). Mouse tracheal epithelial cell (mTEC) cultures were treated with vehicle (EtOH) or P4 and injured or left intact. Relative expression of progesterone receptor (Pr) mRNA was measured every 12 h for 48h (C). Transepithelial resistance (TER) (D), AREG production (E), and Areg mRNA relative expression (F) were measured every 12h for 48h (n = 10/treatment/time-point). Areg and Pr mRNA expression was normalized to Gapdh and to uninjured controls using the ΔΔCt method (n = 5/treatment/time-point). Bars or squares represent means ±SEM from 2–3 independent experiments, with significant differences represented by asterisks (*).

Table 2.1: **Total numbers of CD4+ and CD8+ T cells in lung single cell suspensions from IAV-infected ovariectomized female mice treated with placebo (-P4) or progesterone (+P4) at 7dpi.** Data are presented as the mean ± SEM from four independent experiments (CD8+ T cells: n=6-8/treatment; CD4+ T cells: n=10-12/treatment) and were analyzed by T-tests.

Supplemental Table 2.1. Cytokine and chemokine concentrations in lung homogenates from ovariectomized female mice treated with placebo (-P4) or progesterone (+P4). Adult female mice were ovariectomized, treated with placebo (-P4) or exogenous P4 (+P4), and inoculated with lethal IAV or mock-infected. Supernatant from whole lung homogenates was used to quantify cytokines at 3,5 and 7dpi. Data are presented as the mean ± SEM in pg/ml (a) or ng/ml (b) from two independent experiments (n=8-10/treatment/dpi). Data were analyzed with two-way ANOVA followed by Tukey tests, with significant differences compared with –P4 females at an individual time-point represented by an asterisk (*), significant differences within a treatment group at 7 or 5dpi compared with day 3 represented by a plus (+) $P>0.05$.

Figure 2.1

A

	Progesterone (ng/ml)		Uterine horn mass (mg)	
Treatment	Mock	IAV	Mock	IAV
- P4	3.08±0.8	2.33±0.4	16.88±1.5	14.88±1.6
+ P4	10.42±0.7 *	13.9±0.7 *	30.23±3.0 *	32.77±1.9 *

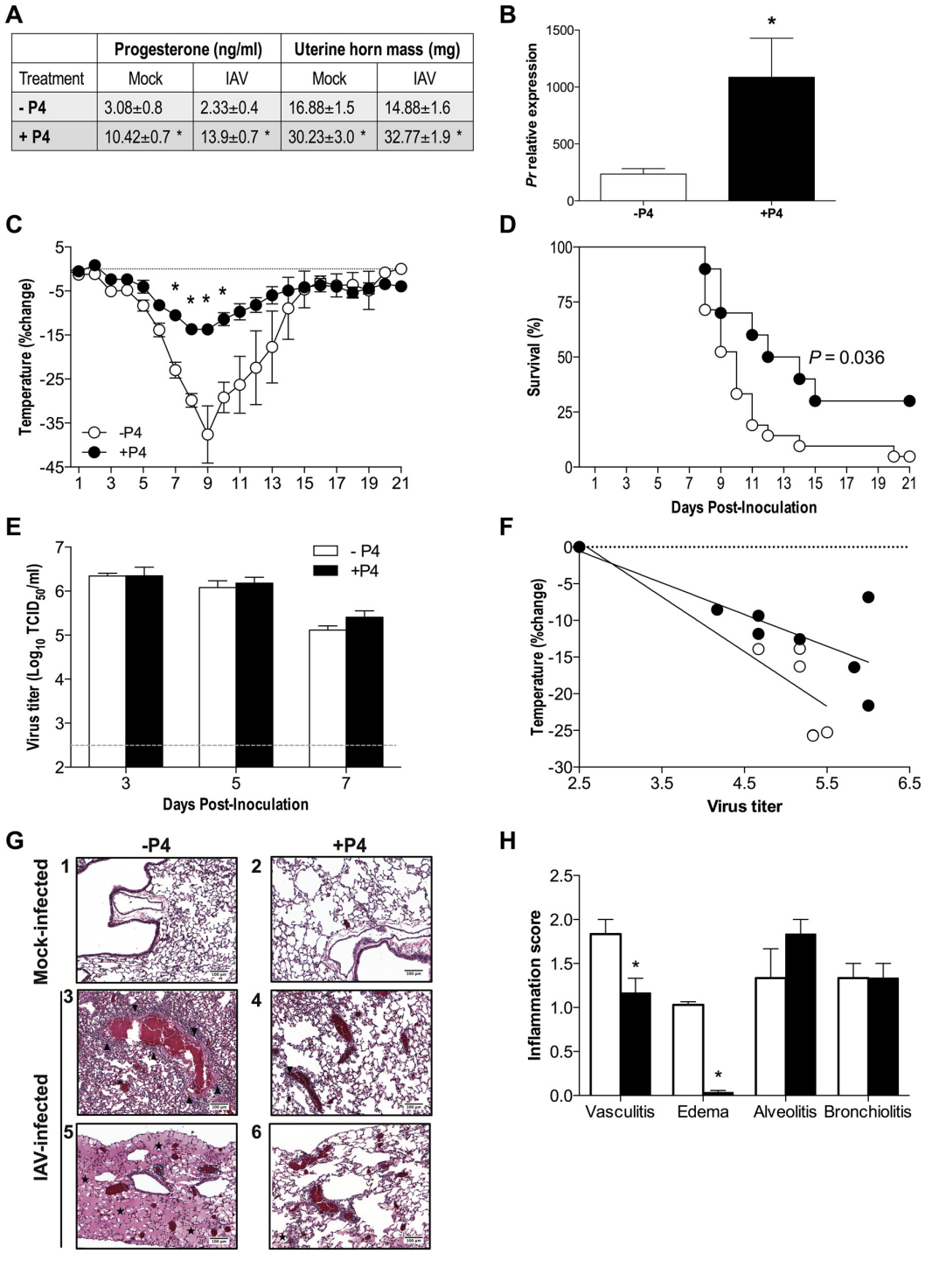

Figure 2.2

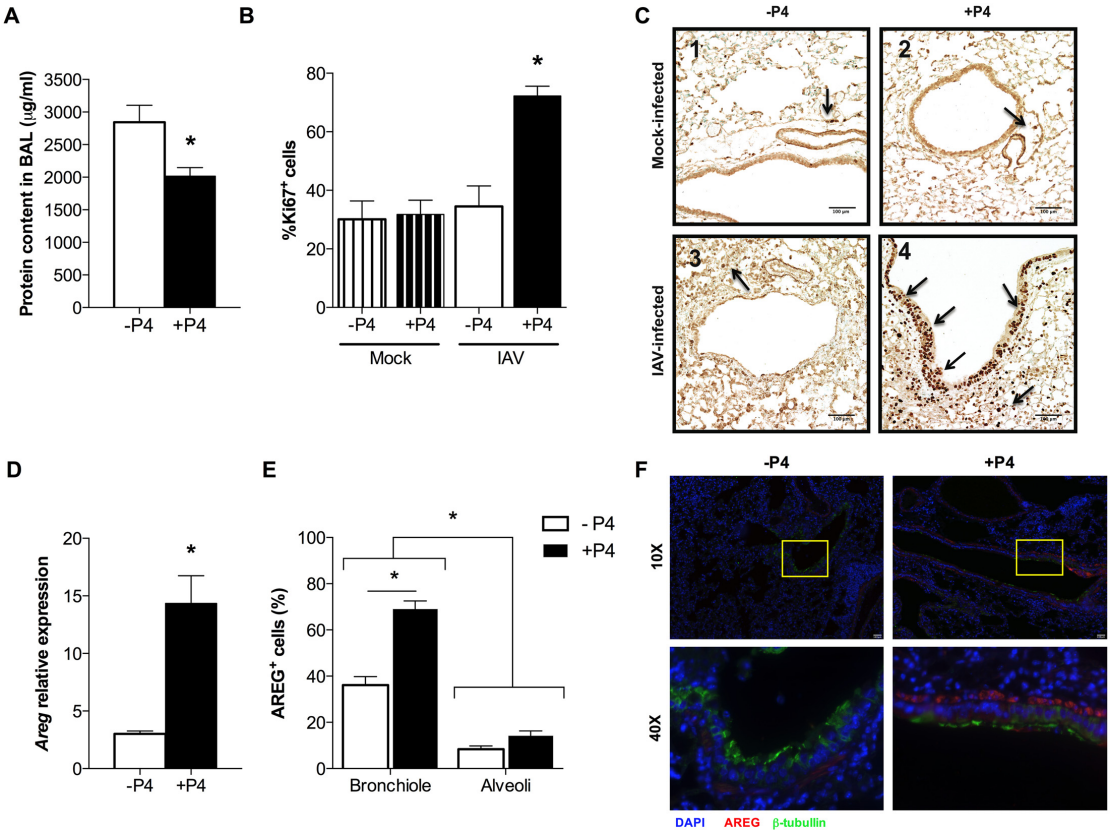
A
Protein content in BAL (μg/ml)
3500
3000
2500
2000
1500
1000
500
0
*
-P4
+P4
B
%Ki67+ cells
80
60
40
20
0
*
-P4
+P4
-P4
+P4
Mock
IAV
C
-P4
+P4
Mock-infected
IAV-infected
1
2
3
4
D
Areg relative expression
20
15
10
5
0
*
-P4
+P4
E
AREG+ cells (%)
100
80
60
40
20
0
*
*
- P4
+P4
Bronchiole
Alveoli
F
-P4
+P4
10X
40X
DAPI
AREG
β-tubullin

Figure 2.3

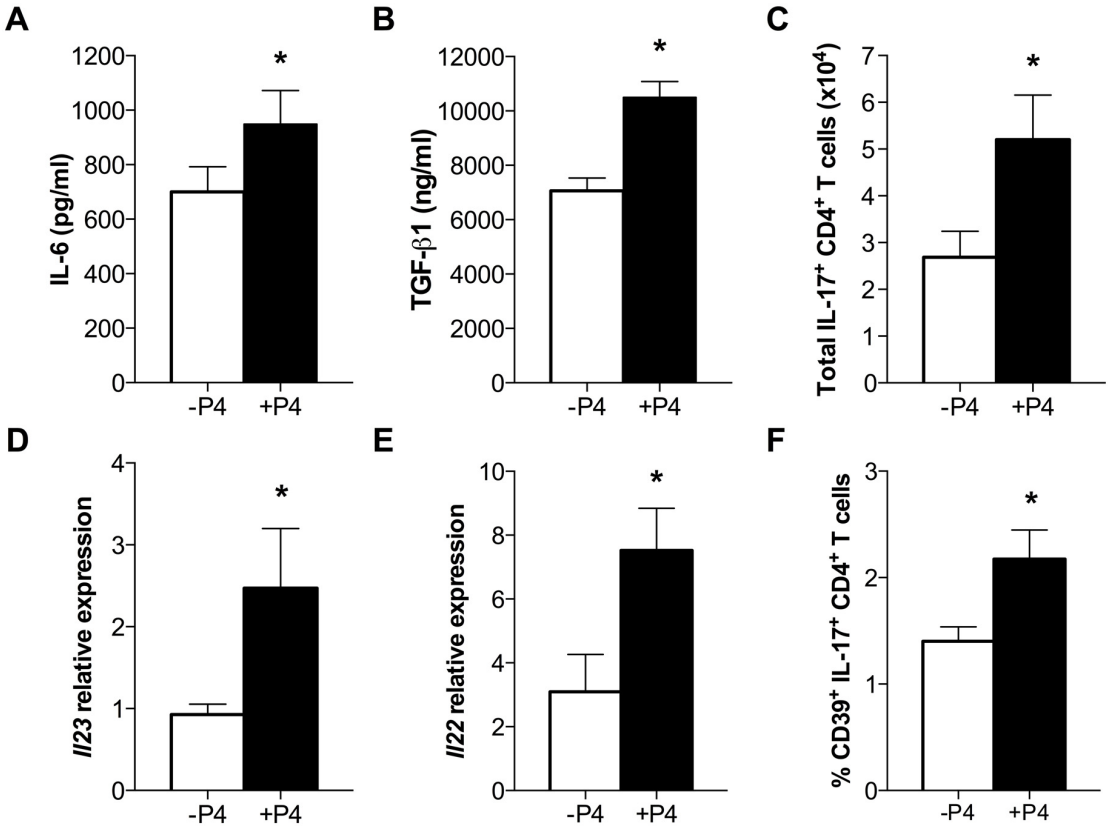
A
IL-6 (pg/ml)
1200
1000
800
600
400
200
0
*
-P4
+P4
B
TGF-β1 (ng/ml)
12000
10000
8000
6000
4000
2000
0
*
-P4
+P4
C
Total IL-17+ CD4+ T cells (x10^4)
7
6
5
4
3
2
1
0
*
-P4
+P4
D
Il23 relative expression
4
3
2
1
0
*
-P4
+P4
E
Il22 relative expression
10
8
6
4
2
0
*
-P4
+P4
F
% CD39+ IL-17+ CD4+ T cells
3
2
1
0
*
-P4
+P4

Figure 2.4

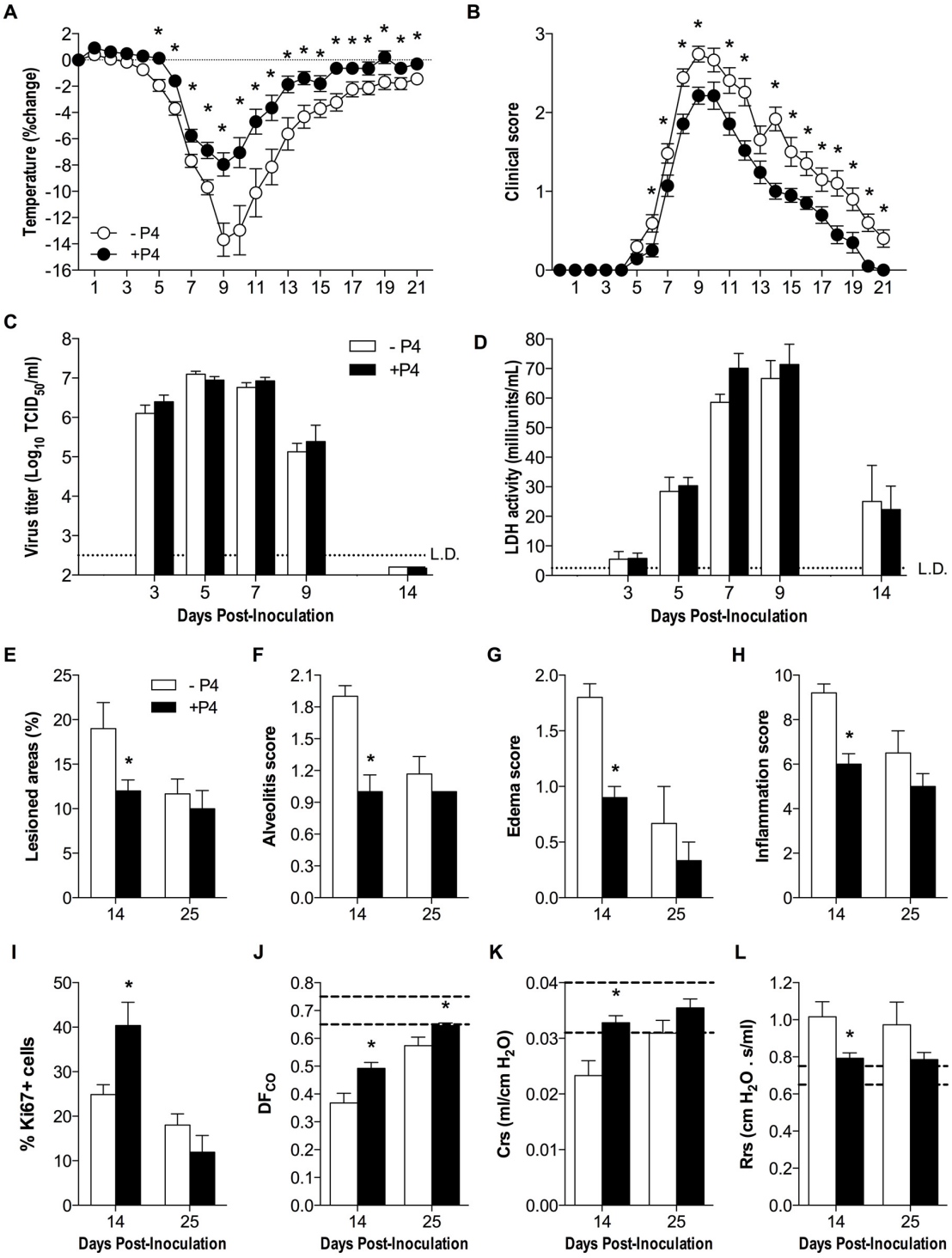
A
Temperature (%change)
- P4
+P4
B
Clinical score
C
Virus titer (Log10 TCID50/ml)
L.D.
Days Post-Inoculation
D
LDH activity (milliunits/mL)
L.D.
Days Post-Inoculation
E
Lesioned areas (%)
F
Alveolitis score
G
Edema score
H
Inflammation score
I
% Ki67+ cells
Days Post-Inoculation
J
DFCO
Days Post-Inoculation
K
Crs (ml/cm H2O)
Days Post-Inoculation
L
Rrs (cm H2O . s/ml)
Days Post-Inoculation

Figure 2.5

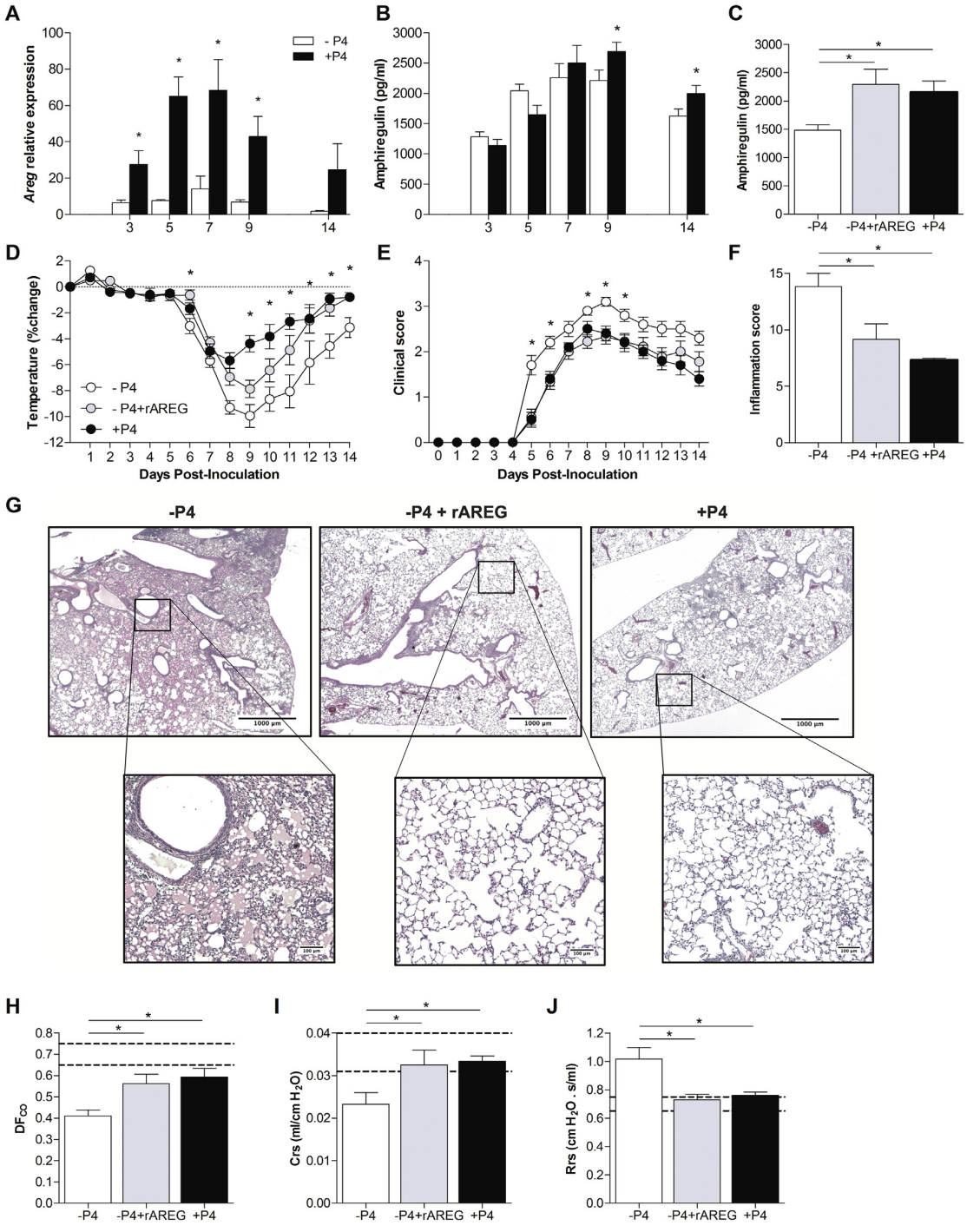
A
Areg relative expression
- P4
+P4
B
Amphiregulin (pg/ml)
C
Amphiregulin (pg/ml)
-P4
-P4+rAREG
+P4
D
Temperature (%change)
- P4
- P4+rAREG
+P4
Days Post-Inoculation
E
Clinical score
Days Post-Inoculation
F
Inflammation score
-P4
-P4 +rAREG
+P4
G
-P4
-P4 + rAREG
+P4
1000 µm
100 µm
H
DFco
I
Crs (ml/cm H2O)
J
Rrs (cm H2O . s/ml)

Figure 2.6

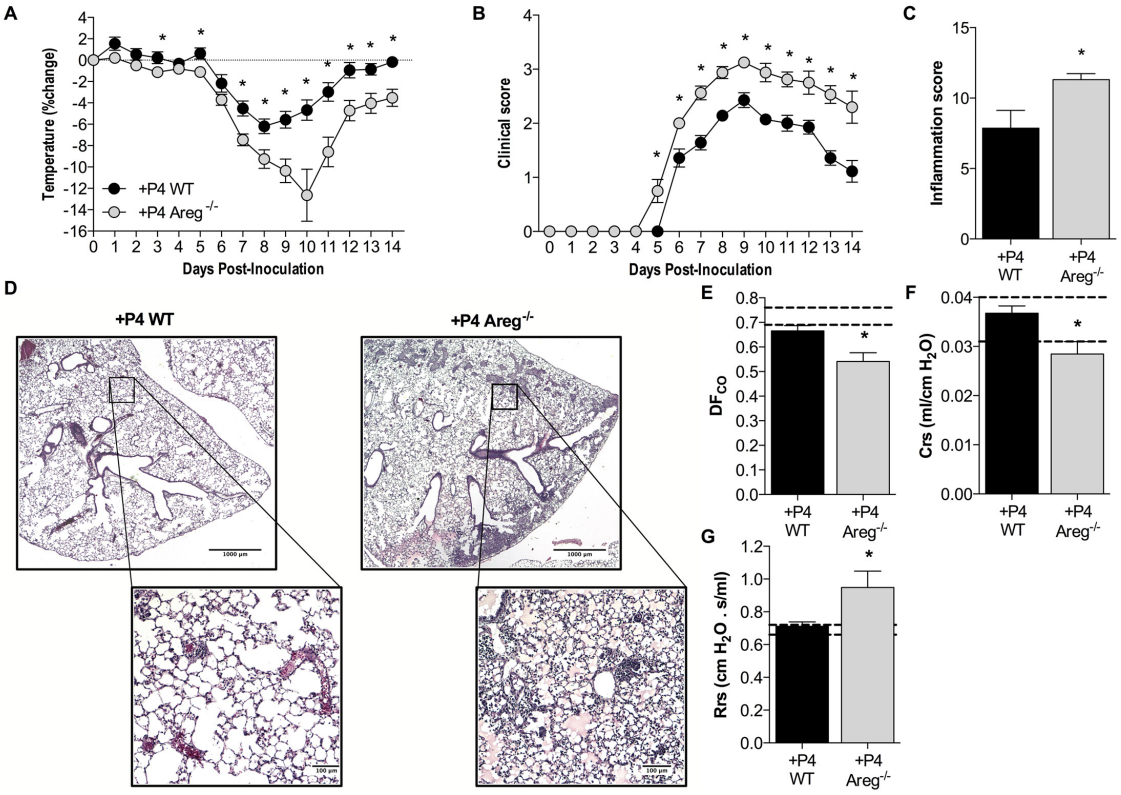
A
Temperature (%change)
Days Post-Inoculation
+P4 WT
+P4 Areg -/-
B
Clinical score
Days Post-Inoculation
C
Inflammation score
+P4 WT
+P4 Areg-/-
D
+P4 WT
+P4 Areg-/-
E
DFco
F
Crs (ml/cm H2O)
G
Rrs (cm H2O . s/ml)

Figure 2.7

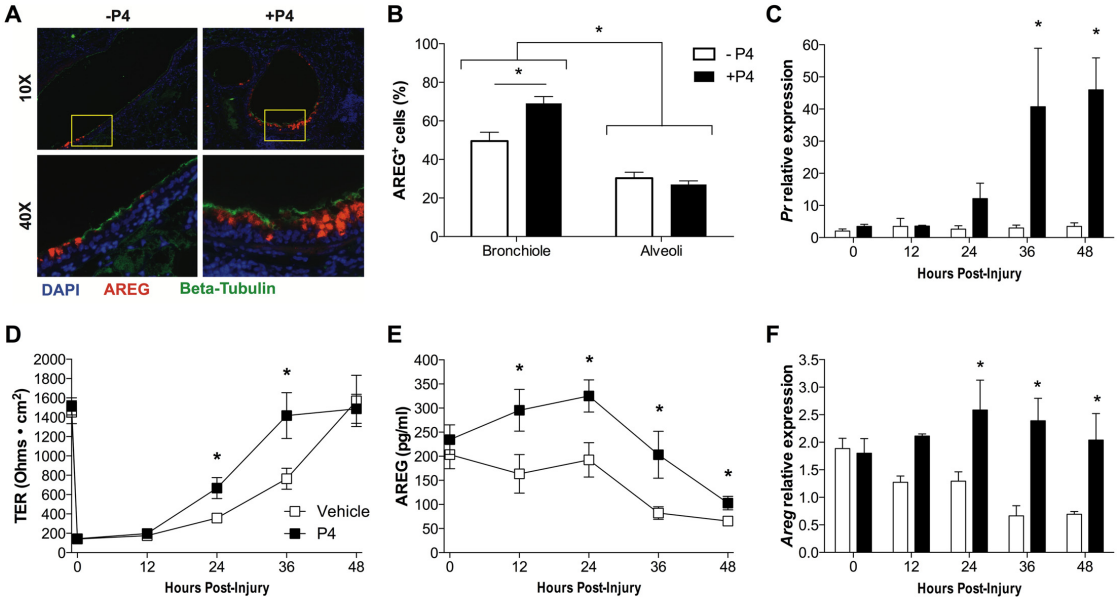
A
-P4
+P4
10X
40X
DAPI
AREG
Beta-Tubulin
B
AREG+ cells (%)
- P4
+P4
Bronchiole
Alveoli
C
Pr relative expression
Hours Post-Injury
D
TER (Ohms • cm2)
Vehicle
P4
Hours Post-Injury
E
AREG (pg/ml)
Hours Post-Injury
F
Areg relative expression
Hours Post-Injury

Table 2.1

Total numbers of cells	**-P4**	**+P4**
CD8+ T cells (x10^5)	2.43±0.52	1.91±0.29
$NP_{366-374}$ CD8+ T cells (x10^4)	5.7±0.49	3.28±0.68
IFN-γ+ CD8+ T cells (x10^4)	1.37±0.6	1.84±0.65
TNF-α CD8+ T cells (x10^4)	7.44±2.2	5.42±1.76
Total CD4+ T cells (x10^5)	4.35±0.73	3.46±0.52
IFN-γ+ CD4+ T cells (x10^4)	2.96±0.91	3.45±1.22
IL4+ CD4+ T cells (x10^4)	2.62±0.75	5.14±1.76
Foxp3+ CD4+ T cells (x10^4)	5.58±2.28	3.47±1.66

Supplemental Table 2.1

Cytokine	Treatment	Days Post-Infection 3	5	7
IL-1β[a]	- P4	928.7±135.2	1308.4±129.7	710.7±110.9
	+P4	1253.9±96.4	1576.1±227.4	1022.9±202.6
TNF-α[a]	- P4	162.7±84.4	235.4±16.2	313.0±21.0
	+P4	140.3±7.4	279.9±20.0	371.3±18.8*
IL-6[a]	- P4	978.3±119.7	1339.2±107.4	700.2±92.5
	+P4	**1207.9±2**[*]	**1478.7±177.3**[*]	**949.0±122.8**[*]
IFN-γ[a]	- P4	82.7±6.4	67.7±4.9	3889.3±457.7[+]
	+P4	56.6±3.65	104.2±13.6	3092.4±399.3[+]
IL-12p70[a]	- P4	6.6±0.4	10.5±0.9[+]	16.4±1.2[+]
	+P4	5.6±0.8	8.4±1.5[+]	17.5±1.6[+]
IL-4[a]	- P4	37.6±10.6	71.6±23.6	52.9±26.5
	+P4	28.6±4.1	30.3±6.4	11.8±3.8
IL-5[a]	- P4	27.9±5.7	57.3±8.9	66.6±9.3
	+P4	42.7±13.9	48.6±9.9	**110.1±10.4***
IL-13[a]	- P4	461.6±27.2	243.2±56.3	184.3±48.6
	+P4	**191.6±42.0***	189.7±40.1	309.7±90.4
IL-33 [b]	- P4	57.6±8.3	41.8±9.0	21.3±5.6
	+P4	41.0±9.9	28.7±5.6	16.9±3.4
IL-10[a]	- P4	25.2±4.5	24.2±3.1	158±17.9[+]
	+P4	26.9±4.4	21.1±2.7	178.5±25.7[+]
TGF-β [b]	- P4	8299.0±1367.0	5953.0±469.0	7433.0±674.0
	+P4	5460.0±921.0	7368.0±1738.0	**10734.0±890.0***

Chapter 3

Progesterone-based contraceptives reduce adaptive immune responses and protection against sequential influenza A virus infection

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Abstract

In addition to their intended use, progesterone (P4)-based contraceptives promote anti-inflammatory immune responses, yet their effects on the outcome of infectious diseases, including influenza A virus (IAV), are rarely evaluated. To evaluate their impact on immune responses to sequential IAV infections, adult female mice were treated with placebo or one of two progestins, P4 or levonorgestrel (LNG), and infected with mouse adapted (ma) H1N1 virus. Treatment with P4 or LNG reduced morbidity during primary H1N1 infection as compared to placebo treatment. In serum and bronchoalveolar lavage fluid, total anti-IAV IgG and IgA titers and virus neutralizing antibody titers, but not hemagglutinin stalk antibody titers, were lower in progestin-treated mice as compared with placebo-treated mice. Females were challenged six weeks later with either a maH1N1 drift variant (maH1N1dv) or maH3N2 IAV. Protection following infection with the maH1N1dv was similar among all groups. In contrast, following challenge with maH3N2, progestin treatment reduced survival as well as numbers and activity of H1N1- and H3N2-specific memory CD8+ T cells, including tissue resident cells, compared with placebo treatment. In contrast to primary IAV infection, progestin treatment increased neutralizing and IgG antibody titers against both challenge viruses compared with placebo treatment. While the immunomodulatory properties of progestins protected naïve females against severe outcome from IAV infection, it made them more susceptible to secondary challenge with a heterologous IAV, despite improving their antibody responses against a secondary IAV infection. Taken together, the immunomodulatory effects of progestins differentially regulate the outcome of infection depending on exposure history.

Importance

The impact of hormone-based contraceptives on the outcome of infectious diseases outside of the reproductive tract is rarely considered. Using a mouse model, we have made the novel observation that treatment with either progesterone or a synthetic analog found in hormonal contraceptives, levonorgestrel, impacts sequential influenza A virus infection, by modulating antibody responses and decreasing memory CD8+ T cells. Progestins reduced antibody responses during primary H1N1 virus infection, but increased antibody titers following a sequential infection with either a H1N1 drift variant or a H3N2 virus. Following challenge with a H3N2 virus, female mice treated with progestins experienced greater mortality with increased pulmonary inflammation and reduced numbers and activity of CD8+ T cell. This study suggests that progestins significantly affect adaptive immune responses to influenza A virus infection, with their effect on influence outcome depending on exposure history.

Introduction

Adults worldwide are exposed to multiple influenza viruses and viral antigens during their lifetime both through natural infection and vaccination. In the United States, since 2010, vaccination has been recommended for all individuals ages 6 months and older (317). By six years of age, all children have been exposed to at least one influenza virus, with infections with novel antigenically distinct viruses occurring every 5-10 years (263, 318). By adulthood, most individuals are no longer influenza-naïve, which is rarely considered in animal models of influenza A virus (IAV) pathogenesis.

Protective immune responses to sequential IAV infections include cross-reactive antibodies, including broadly neutralizing antibodies which target the conserved stalk region of the hemagglutinin (HA) antigen and CD8+ T cell responses, the latter being the primary mechanism of cross-protection against heterosubtypic influenza strains, as CD8+ T cells can recognize conserved viral proteins, including the nucleoprotein (180, 204, 205). In addition to circulating memory T cells and T central memory (TCM) cells that traffic through the lymph nodes, tissue-resident memory (TRM) cells promote local and immediate protection in the lungs with the ability to expand rapidly, kill virus-infected cells, recruit circulating memory T cells, and release cytokines, resulting in TRMs being indispensable for heterosubtypic protection (206-208).

Several factors, including the sex, age, and reproductive status of the host can influence adaptive immune responses and the outcome of IAV infection (211, 319, 320). During IAV infection, sex steroids, in general, and progesterone (P4), in particular, can alter the functioning of immune cells and respiratory epithelial cells to reduce pulmonary inflammation, improve pulmonary repair and function, and cause faster recovery from primary infection with IAV (131, 320, 321). Data from our lab (131) and others (33, 34, 39, 53, 60) illustrate that P4 has broad anti-inflammatory properties, resulting in reduced activity of NK and T cells, lower antibody

responses, greater concentrations of TGF-β, and increased numbers and activity of regulatory T cells and regulatory Th17 cells.

In addition to natural exposure to P4 through ovarian and placental production during reproductive cycles and pregnancy, respectively, females can be exposed to synthetic forms of P4 (i.e., progestins) through the use of hormone-based contraceptives, all of which contain some form of progestin (4). It is estimated that 88% of all adult women in the US have been exposed to progestins in some form of contraceptives (20). Progestins, such as levonorgestrel (LNG), are more commonly used in contraceptives than P4 because they bind to the progesterone receptor with greater affinity, have a longer half-life, and cause fewer side-effects (19). Despite the broad use of progestins and their known anti-inflammatory properties, their effects on viral infections at mucosal sites outside of the reproductive tract have not been adequately explored.

The goal of this study was to evaluate the effects of both P4 and LNG on adaptive immune responses and the outcome of sequential infection with an IAV drift variant or heterosubtypic IAV. We hypothesized that, similar to the effects of P4 treatment in ovariectomized mice, exposure to either P4 or LNG in ovary-intact mice would improve the outcome of a primary IAV infection in immunologically naïve females by reducing inflammation and adaptive immune responses that contribute to immune-mediated pathology (131). Following secondary challenge with either a drift variant or heterosubtypic strain of IAV, we speculated that the reduced adaptive immune responses to the primary infection, while beneficial for recovery from the primary infection, may cause reduced protection against a subsequent infection. Our findings demonstrate that following treatment with either P4 or LNG, females were better protected from a primary IAV infection, despite generating lower antibody and memory CD8+ T cell responses. Following secondary infection with a heterosubtypic, but not a drift variant IAV virus, females treated with either P4 or LNG suffered a worse disease outcome from infection,

which was likely mediated by insufficient memory T cell responses as opposed to antibody responses from the primary infection.

Results

Progesterone and levonorgestrel protect against primary IAV infection, but reduce systemic and pulmonary antibody responses against IAV

Adult female mice were treated with subcutaneous placebo, P4, or LNG pellets, which delivered a continuous dose of hormone over the course of 60 days, and infected with a low dose of ma2009 H1N1 virus. Similar to previously published results in ovariectomized female mice (131), treatment of ovary-intact females with P4 reduced morbidity following IAV infection, by decreasing body mass loss, hypothermia, and clinical disease as compared to placebo treatment (**Fig. 3.1A** and **B** and data not shown; $P<0.05$). One of the most common progestins (i.e., synthetic analog of P4) used in hormonal contraceptives is LNG, which signals through the progesterone receptor with greater affinity than P4 (11). Similar to P4 treatment, mice treated with LNG and infected with a low dose of ma2009 H1N1 virus had less morbidity than placebo-treated females during infection, including reduced body mass loss, hypothermia, and clinical signs (**Fig. 3.1A** and **B** and data not shown; $P<0.05$).

Serum titers of anti-ma2009 IgG antibodies were measured at 14, 21, 28, and 35 days post-inoculation (dpi) and did not differ between placebo- and P4-treated females, but were transiently lower at 21 dpi following LNG treatment (**Fig. 3.1C**; $P<0.05$). Serum titers of neutralizing antibodies measured at 14, 21, 28, and 35 dpi against the ma2009 virus were significantly reduced in females with P4 (at 21 dpi) or LNG (at 14 and 35 dpi) treatment as compared to placebo (**Fig. 3.1D**; $P<0.05$). Progestins reduced neutralizing antibody responses, however, titers at 35 dpi remained around the 1/640 dilution, which is high enough to protect mice against a homologous challenge (218). Antibody titers in the bronchoalveolar lavage (BAL) fluid were also analyzed at 14, 21 and 28 dpi to assess the mucosal immune response at the local site of infection. Both anti-ma2009 IgA and neutralizing antibody titers against ma2009 in the BAL were reduced in females treated with either P4 (at 28 dpi) or LNG (at 21 and 28 dpi) as

compared to females treated with placebo (**Fig. 3.1E** and **F**; $P<0.05$). In summary, P4 and, to a greater extent, LNG protect females against IAV infection, but significantly reduce systemic and local antibody production during a primary infection.

Protection against an H1N1 drift variant and hemagglutinin stalk antibody responses are not affected by treatment with progestins

To assess whether the P4 and LNG-induced reduction in serum and, to a greater extent, BAL antibody titers (**Fig. 3.1C-F**) could impact the outcome of sequential infection with a closely related H1N1 virus, female mice were treated with P4, LNG, or placebo, inoculated with ma2009 H1N1 virus, and then challenged six weeks later with a H1N1 ma2009 drift variant (ma2009dv) which contained a mutation at position 166 in the HA head domain (K166Q) (145) (**Fig. 3.2A**). All females, regardless of hormone treatment, were equally protected against the ma2009dv H1N1 (**Fig. 3.2B**). Hemagglutinin stalk-specific antibodies recognize the conserved stalk region of the HA protein of IAVs and contribute to cross protection against diverse IAVs (178, 181). To assess whether titers of broadly neutralizing antibodies against the stalk of H1 may contribute to the similar level of protection against the ma2009dv observed in both placebo- and hormone-treated females following challenge, we measured stalk-specific antibody responses in serum at several time-points following the primary ma2009 H1N1 virus infection. Stalk antibody titers increased over time, peaking at 28 dpi in all female mice, and did not differ between placebo-treated and either P4- or LNG-treated females (**Fig. 3.2C**).

To assess whether the P4-based treatments affected antibodies against the primary virus following the ma2009dv challenge, we measured serum anti-ma2009 neutralizing antibody and IgG titers pre- and post-challenge with ma2009dv. Following challenge with a drift variant virus, antibody responses to the primary virus were boosted at 21 days post-challenge in all treatment groups (**Fig. 3.2D** and **E**, $P<0.05$). Among all treatment groups, despite minimal pre-existing serum neutralizing antibody and IgG titers to the drift variant, following challenge with

maH1N1dv, serum neutralizing antibodies to the drift variant were detectable as early as two days post challenge and were associated with complete clearance of the virus from the lungs (**Fig. 3.2F** and data not shown). Serum anti-IAV IgG antibody responses against the ma2009dv also increased as early as two days post-challenge, but were lower in the placebo-treated as compared to P4- or LNG-treated females at 21 days post-challenge (**Fig. 2G**; $P<0.05$). Taken together, these data suggest that P4-based treatments did not alter broadly neutralizing stalk antibody responses or protection against a drift variant of H1N1, but increased total IgG antibody responses following challenge with ma2009dv.

Progestins reduce protection against a heterologous IAV challenge while increasing antibody titers to the challenge virus

Treatment with either P4 or LNG did not affect the outcome of challenge with a homologous group 1 drift variant (**Fig. 3.2B**), but during influenza seasons both H1N1 and H3N2 subtypes circulate. H1 and H3 IAVs are antigenically distinct, belonging to group 1 and group 2 HA phylogenetic groups, respectively (322). To analyze the effects of progestins on a heterologous IAV challenge with a HA group 2 IAV, female mice were treated with either P4, LNG, or placebo, infected with ma2009 H1N1 virus, and challenged six weeks later with a maH3N2 virus, HK68, at a dose that is uniformly lethal in naïve animals (**Fig. 3.3A**). In contrast to primary IAV infection, placebo-treated females were protected against lethal maH3N2 virus challenge with 87% (13/15) of placebo-treated females surviving the challenge as compared to 53% (8/15) of P4- and 43% (6/14) of LNG-treated females surviving infection (**Fig. 3.3B**; $P<0.05$). In the mice that survived the challenge, however, progestin treatment promoted faster recovery as compared to placebo treatment (**Fig.3.3C**; $P<0.05$), which is consistent with the effects during a primary infection (**Fig. 3.1A and B**). Treatment with either P4 or LNG did not alter H3N2 titers or clearance in the lungs (**Fig. 3.3D**).

To determine whether humoral immune responses during the heterosubtypic challenge were affected by progestins in the mice that survived the challenge, neutralizing and total IgG antibody responses against the primary ma2009 IAV and the secondary and heterologous HK68 IAV were measured both prior to and after challenge. In contrast to the ma2009dv challenge (**Fig. 3.2D**), when females were challenged with a heterosubtypic maH3N2 the titers of neutralizing antibody against the primary H1N1 IAV (i.e., ma2009) were not altered (**Fig. 3.3E**). Pre-existing serum neutralizing antibodies to HK68 were not detected in the serum prior to the challenge in any of the treatment groups (**Fig. 3.3F**), but titers against HK68 were significantly greater in P4- and LNG-treated females compared with placebo-treated females at 14 and 21 days post-challenge (**Fig. 3.3F**; $P < 0.05$). Similarly, serum anti-HK68 IgG antibody titers were not detected prior to challenge, but were significantly greater at 21 days post-challenge in both P4- and LNG-treated mice than in placebo-treated females (**Fig. 3.3G**; $P<0.05$). Although treatment with progestins reduced neutralizing antibody responses to primary IAV infection, these hormones significantly increased neutralizing antibody responses against a sequential infection with an antigenically unrelated IAV. Taken together, these data illustrate that in the context of a heterologous challenge, progestins increase antibody responses to sequential influenza A virus infection, without having an effect on antibodies against the primary virus, which may be beneficial for vaccine responses.

Progestins increase pulmonary immunopathology following challenge with a heterologous IAV

Because titers of HK68 were not different between placebo and progestin treated females (**Fig. 3.3D)**, we sought to identify other possible mechanisms of differential mortality between the placebo- and progestin-treated females following a heterologous challenge. We analyzed pulmonary immunopathology in female mice that were treated with either P4, LNG, or placebo, infected with ma2009 H1N1 virus, and challenged six weeks later with a maH3N2 virus. Lungs

were inflated and fixed at 6 and 8 days post-challenge to encompass the average day of death for females in each of the progestin treatment groups (average day of death for P4: 7.8±0.7, and LNG: 6.6±0.2). No differences in overall inflammation scores were observed at day 6 post H3N2 challenge across the treatment groups, but by day 8 post-challenge, females treated with either P4 or LNG had greater inflammation, as characterized by increased alveolitis, necrosis, and edema as compared to placebo-treated females (**Fig. 3.4A-C**: $P<0.05$). Taken together, these data indicate that the increased mortality following a heterologous H3N2 challenge in progestin-treated females may be caused by excessive immunopathology in the lungs at the time of death.

Progestins reduce memory CD8+ T cell responses against a heterologous IAV challenge

Although antibody responses to the primary ma2009 H1N1 infection were significantly lower in P4- and LNG-treated females compared with placebo-treated females, antibody-mediated immunity is not the primary mechanism of cross-protection against different groups of IAVs which possess distinct surface antigens, but share common core proteins that could be recognized by T cells. Populations of memory T cells mediate cross-protection against different groups of IAVs (180, 203-205) and were analyzed at several time points prior to and after challenge with the H3N2 IAV to determine if suppressed memory T cell numbers and activity in progestin-treated females may underlie the increased susceptibility to heterosubtypic infection. Treatment with either P4 or LNG did not alter numbers of total virus-specific CD4+ T cell, Th2 cell, or Th17 cell subsets in the lungs but reduced numbers of Th1 cells after challenge (**Table 3.1**). Frequencies of total CD8+ T cells and naïve CD8+ T cells also were not significantly different between placebo-, P4-, and LNG-treated mice prior to or after heterosubtypic IAV challenge (**Table 3.1**).

Following challenge with a heterosubtypic maH3N2, the frequency of most virus-specific CD8+ T cell populations in the lungs increased over time, regardless of hormone treatment (**Fig.**

3.5A-D). Treatment of females with either P4 or LNG, significantly reduced the total number of ma2009-specific CD8+ T cells producing IFN-γ, TNF-α, or both cytokines, as compared to placebo treatment at 6 days post-challenge (**Fig. 3.5A-C**; $P<0.05$). Tetramer-specific CD8+ T cells, that recognized either the ma2009- (i.e., primary IAV) or HK68- (i.e., secondary IAV) specific immunodominant NP peptides, also were lower at 6 days post-challenge in females treated with either P4 or LNG as compared to placebo (**Fig. 3.5D**; $P<0.05$).

Total numbers of CD8+ memory ($CD44^+$) T cells, following *ex vivo* stimulation with a peptide from ma2009, were also decreased 6 days following heterosubtypic IAV challenge in the females treated with either P4 or LNG as compared to placebo (**Fig. 3.6A**). This included both CD8+ tissue resident memory (TRM; $CD8+CD44^+CD69^+CD103^+$), and CD8+ T central memory (TCM; $CD8+CD44^+CD62L^+$) cells (**Fig. 3.6B-D**; $P<0.05$). The total number of virus-specific memory CD8+ T cells producing IFN-γ with or without TNF-α at days 2 and 6 post-challenge, and TNF-α producing memory CD8+ T cells at day 6 post-challenge were also significantly decreased in progestin-treated females as compared to females treated with placebo (**Fig. 3.6E-G**; $P<0.05$). Similarly, when memory CD8+ T cells were stimulated *ex vivo* with a HK68 peptide, the numbers of TNF-α+ producing CD8+ T cells, IFN-γ+TNF-α+ producing CD8+ T cells, memory CD8+ T cells, CD8+ TRM cells, and CD8+ TCM cells were lower in the lungs of P4- and LNG-treated females than placebo-treated females at 2, 6, or both days post-challenge (**Table 3.2**; $P<0.05$). Taken together, these data illustrate that IAV-specific memory CD8+ T cell numbers and activity, but not total CD8+ or CD4+ T cell numbers, were significantly reduced by progestin treatment during the heterosubtypic challenge.

Discussion

A significant majority of women in the United States are exposed to some form of progestin in either hormone contraceptives or hormone-replacement therapy (20). Concurrently, women (and men) are exposed to novel strains of IAVs approximately every 5-10 years over the life course (318). The impact of progestins on the outcome and responses to infectious diseases at mucosal sites outside of the reproductive tract are rarely considered. In the present study, we show that while both P4 and LNG improved the outcome of IAV in immunologically naïve female mice, these hormones significantly reduced memory CD8+ T cell responses and thereby increased susceptibility to heterosubtypic challenge with novel strains of IAV. Surprisingly, and possibly beneficial for vaccine responses, both P4 and LNG improved antibody responses to subsequent IAV challenges.

Following H1N1 infection and subsequent challenge with an H1N1 drift variant, placebo-, P4-, and LNG-treated females were equally protected, despite low pre-existing antibody responses to the ma2009dv, and reduced antibody responses to the primary H1N1 infection in progestin-treated females. The K166Q mutation is located in the globular head region of the HA, with the stalk regions of both the ma2009 H1N1 and the ma2009dv H1N1 being conserved (145). To address whether the protection following a drift variant virus challenge was due to antibody responses to the stalk region of the HA, we measured broadly neutralizing antibody responses to the conserved stalk region of the H1 HA using a chimeric HA containing an exotic H6 head and the stalk from the ma2009 H1N1 HA. Broadly neutralizing antibodies target epitopes within the HA stem region that are conserved within each phylogenetic HA group. In our study, broadly neutralizing stalk antibody responses were not affected by hormone treatment, which may explain why there was equal protection against another group 1 virus and complete clearance of the virus by two days post-challenge (178, 323). Additionally, the neutralizing antibody response to the

drift variant was boosted as early as two days post-challenge with the drift variant virus and may explain why these females were fully protected.

Following H1N1 infection and subsequent challenge with a heterologous H3N2 strain of IAV, females treated with either P4 or LNG suffered greater mortality between 6 and 8 days post-challenge. The increased susceptibility to the secondary heterologous IAV challenge was not caused by effects of either P4 or LNG on virus replication or clearance, but was associated with increased pulmonary immunopathology, including edema, alveolitis and necrosis, and impaired memory CD8+ T cell responses. Memory CD8 T cells are indispensable in the control of heterosubtypic IAV infection, and TRM cells, in particular, are the primary mediator of protection against secondary IAV challenge. Studies in mice show that if the entry of any non-resident, circulating, memory T cells to the lungs is blocked, then the presence of tissue-embedded TRM alone is sufficient to induce heterosubtypic immunity (208). In the current study, reduced virus-specific memory CD8+ T cell and CD8+ TRM cell activity were associated with an inability to survive heterosubtypic IAV challenge in P4 or LNG treated mice. Although the total numbers of pulmonary CD4+ or CD8+ T following H3N2 challenged females were not affected by progestins, the expansion of virus-specific CD8+ T cells that recognize both H1N1 and H3N2 IAVs was restricted in progestin- compared to placebo-treated females. Furthermore, expansion of memory CD8+ T cell subsets occurred following stimulation *ex vivo* with either the H1N1 or H3N2 NP immunodominant peptide, which despite a three amino acid difference, is conserved across group 1 and group 2 viruses and elicit similar memory CD8+ T cell responses.

The ability of CD8+ T cells to rapidly expand and produce anti-viral cytokines, including TNF-α and IFN-γ which can have synergistic effects, is crucial for both the clearance of the virus and the resolution of inflammation (324, 325). Mice with IFN-γ deficient CD8+ T cells or complete TNF-α knock out mice have extensive inflammation and lung pathology with impairment of lung function following IAV infection, demonstrating the importance of these

cytokines not only in the clearance of the virus but also in resolution of infection-induced lung pathology (264, 326). The production of TNF-α and IFN-γ by effector and memory CD8+ T cells in response to heterosubtypic IAV challenge was significantly impaired in females treated with either P4 or LNG. Consequently, both P4 and LNG treated females had increased pulmonary inflammation and lung pathology immediately following heterosubtypic IAV challenge. Progestins may prevent the production of IFN-γ and TNF-α by IAV-specific CD8+ T cells by inhibiting signaling pathways in these cells. When bound to the progesterone receptor, progestins can directly interfere with both the NF-κB and MAPK pathways, which control the transcription of TNF-α and IFN-γ (38, 43, 327, 328), suggesting a potential mechanism for the progestin-mediated reduction of IFN-γ and TNF-α within IAV-specific CD8+ T cells.

The impact of progestins on sequential exposure and memory responses to viral infection have only been evaluated in a few vaccine studies, mostly in the context of herpes simplex virus (HSV) infection. Following HSV-2 vaccination, progestin treatment decreases protection against a challenge, reduces virus-specific IgG and IgA antibody levels, and increases virus shedding (96, 113). Several studies illustrate that progestins inhibit antibody production (60, 61), but most studies only consider this effect after an exposure to antigen in immunologically naïve animals. Consistent with these findings, in the current study, progestins significantly reduced titers of neutralizing and virus-specific IgG and IgA both in the serum and BAL in naïve females infected with IAV. A secondary virus infection with a drift variant virus resulted in a boost in titers to the primary virus in all treatment groups. However, both neutralizing and total IgG antibodies against the secondary challenge virus were significantly higher in P4 and LNG-treated females compared with placebo-treated females. Following secondary challenge with a drift variant of H1N1, progestin treatment reduced antibody responses to a primary IAV infection and increased antibody responses to a secondary IAV challenge as compared with placebo treatment, which may be beneficial in the context of vaccination.

The current usage of hormonal contraceptives is on the rise, with over 100 million women worldwide taking some form of progestin-based hormonal contraceptives. These hormone-based contraceptives are listed by the World Health Organization (WHO) as an essential medication because of their role in preventing excessive pregnancies and maternal morbidity. Additionally, in light of concerns about infectious diseases that can be transmitted from mother to fetus (e.g., Zika virus), the WHO, the Center for Disease Control in the United States, and other national health agencies recommend the use of hormonal contraceptives to prevent pregnancies and transmission of infections to the fetus. In this study, we demonstrate that the exposure to progestins in ovary-intact female mice, has beneficial effects on primary infection with IAV, but detrimental effects on the generation and activation of memory CD8 T cell responses and outcome of secondary infection. The impact of these responses in humans should be considered in human surveillance studies as well as vaccine trials to determine if contraceptives alter the outcome of IAV infection and responses to vaccination in women.

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Materials and Methods

Animals

Adult (7-9 weeks old) female C57BL/6 mice were purchased from Charles River Laboratories (Frederick, MD) and housed up to 5 per microisolator cages under standard BSL-2 housing condition with food and water ad libitum. All animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee under animal protocol M015H236. At 8-12 weeks of age, mice were anesthetized with an intramuscular injection of ketamine (80 mg/kg) and xylazine (8 mg/kg) cocktail and hormones were administered in the form of a subcutaneous dorsal implant: 1) 15 mg progesterone (P4) 60-day release pellets (Innovative Research of America); 2) 5 mm levonorgestrel (LNG; Sigma) in a silastic capsule (0.040 i.d., 0.085 o.d.); or 3) placebo as an empty silastic capsule. The capsules were equilibrated in sterile physiological saline at 37^{o}C overnight prior to implantation. The doses of P4 and LNG increase P4 concentrations, uterine horn mass, or both to within the physiological range of young nonpregnant female mice (131, 300, 301).

Virus Infection, Quantification and Purification

Mouse-adapted influenza A viruses, A/California/04/09 (ma2009;H1N1) generated by Dr. Andrew Pekosz from a published sequence (251) A/California/04/09 drift variant (ma2009dv; H1N1) containing the K166Q mutation of the HA sequence generated by reverse genetics. Viral RNA was extracted (QIAamp Viral RNA, Qiagen) from existing ma2009 IAV and transcribed into cDNA (SuperScript III, Invitrogen). HA specific cDNA was PCR amplified and purified on a 1% agarose gel. Purified HA cDNA was digested and cloned into the pHH21 plasmid vector (329). The K166Q HA mutation (145) was introduced by site directed mutagenesis (QuikChange Lightning Site-Directed Mutagenesis Kit, Agilent Technologies). Viruses encoding the K166Q HA mutation were generated entirely from cDNA using the 12 plasmid rescue system (329, 330). The HA sequence of the rescued virus was confirmed by sequencing the coding region of the HA

gene. Viral stocks were generated by infecting MDCK cells at a multiplicity of infection of 0.01 and the infected cell supernatant was collected at 72 hours post-infection. A/Hong Kong/2/68 (HK68; H3N2) was given to us courtesy of Innocent N. Mbawuike. All three viruses were used in these studies and infectious virus titers were determined using the 50% tissue culture infectious dose ($TCID_{50}$) assay. For infections, mice were anesthetized with a ketamine (80 mg/kg) and xylazine (8 mg/kg) cocktail and inoculated intranasally with 30 μl of a low dose of ma2009 virus (0.04 50% mouse lethal dose [MLD_{50}]) or mock-infected with DMEM alone. For challenge experiments, animals were inoculated with 30 μl of a lethal dose of HK68 (162 MLD_{50} in 30 μl of DMEM) or ma2009drift variant (32 MLD_{50}) 6 weeks following the initial infection.

For virus quantification, a $TCID_{50}$ assay was used in which, log_{10} dilutions of lung homogenates were plated onto a monolayer of Madin-Darby canine kidney (MDCK) cells in replicates of 6 for 6 days at 32°C. Cells were stained with naphthol blue black (Sigma Aldrich) and scored for cytopathic effects. The $TCID_{50}$ titer was calculated according to the Reed-Muench method. For virus purification, viruses were grown in MDCK cells at 37°C for 4 days and pelleted by centrifugation on a 20% sucrose gradient in a Beckman SW28TI rotor at 26,000rpm for 1h at 4°C. The virus pellet was resuspended in 1X PBS, protein was quantified by a BCA assay (Pierce) and aliquots were stored at -80°C.

Morbidity and Mortality Studies

Clinical scores as well as body mass, rectal temperature and survival were recorded daily over the course of the study. Clinical disease scores for IAV-infected mice were based on four parameters, with one point given for each of the following: dyspnea, piloerection, hunched posture and absence of an escape response (131).

Serum and Bronchoalveolar lavage Sample Collection

Serum and bronchoalveolar lavage (BAL) were collected at relevant time-points to measure

antibody titers and neutralizing antibodies. Mice were bled from the cheek for survival experiments or from the retro-orbital sinus for terminal procedures. Serum was heat-inactivated at 56^{o}C for 30min and stored at -80^{o}C. For BAL collection, mice were euthanized by cervical dislocation and the lungs were lavaged twice with 0.5ml of a 0.9% saline solution. BAL fluid was centrifuged at 500g for 10 minutes to remove cells and debris and the supernatant was collected, heat inactivated at 56^{o}C for 30min and stored at -80^{o}C.

Histopathology and Immunohistochemistry

Lungs were inflated at constant pressure, fixed in Z-fix (Anatech) for at least 48h, embedded in paraffin, cut into 5μm sections, and mounted on glass slides. Tissue sections were stained with hematoxylin and eosin (H&E) and used to evaluate lung inflammation. Histopathological scoring was performed by a single blinded veterinary pathologist on a scale from 0-3 (0, no inflammation; 1, mild inflammation; 2, moderate inflammation; and 3, severe inflammation) for the following parameters: bronchiolitis, alveolitis, vasculitis, perivasculitis, necrosis, consolidation, and edema (131). The percentage of lesioned areas within each tissue section was also evaluated. Images were taken using a Nikon Eclipse E800.

Antibody Neutralization

Serially-diluted serum was mixed with 100TCID$_{50}$ of virus (ma2009 or HK68) for 1h at room temperature and used to infect quadruplicates wells of confluent MDC cells for 24h at 37°C. After 16-18h of incubation, the inoculum was removed and the cells washed with 1X PBS (with calcium and magnesium) and fresh media was added. The cells were incubated for 6 days at 32^{o}C and then fixed with 4% formaldehyde and stained with napthtol blue black for 6h. The titer was calculated as the highest serum dilution that eliminated virus cytopathic effects in 2 out of 4 wells per dilution.

Anti-influenza ELISA

ELISA plates (Microlon 96 well high binding plates; Greiner Bio-One) were coated with 100ng of purified virus overnight at 4°C in carbonate buffer (pH=9.6). For IgG ELISAs, plates were washed 3 times with PBST (1X PBS + 0.1% Tween-20 (Sigma)) and blocked for at least 1h at 37°C with 10% dry milk powder in 1X PBS. Plates were washed 3 times prior to the addition of serially diluted serum to the plates for 1h at 37°C at a starting dilutions of 1:1000. Anti-mouse horseradish peroxidase (HRP) conjugated secondary IgG (1:5000; Thermo) was added and plates were incubated for 1 h at 37°C. The plates were washed 3 times with PBST and reactions were developed with 3,3',5,5' tetramethylbenzidine (TMB; BD Biosciences) and stopped using 1N HCl. Plates were read at 450 nm absorbance on a plate reader. For IgA ELISAs, plates were washed 3 times with TBST (1X TBS + 0.1% Tween) and blocked for at least 1h at 37°C with 10% dry milk powder in 1X TBS. Plates were washed 3 times prior to the addition of serially diluted BAL to the plates for 1h at 37°C at a starting dilutions of 1:1. Anti-mouse alkaline phosphatase (AP) secondary IgA (1:2000; Southern Biotech) was added and plates were incubated for 1 h at 37°C. The plates were washed 3 times with TBST and reactions were developed with p-nitrophenyl phosphate substrate (PNPP; Thermo) and stopped using 2N NaOH. Plates were read at 405nm absorbance on a plate reader. To determine the antibody titer, a cutoff value was determined by multiplying the average OD values of the negative controls at each dilution by 3. The titer for the sample was calculated as the highest serum dilution with an OD value above the cutoff.

Stalk antibody ELISA

Flat bottom Immuno 4HBX 96-well plates (Thermo) were coated overnight at 4 °C with 100ng of recombinant HA using the chimeric cH6/1 protein (with the A/mallard/Sweden/81/02 H6 head and a A/Cal/04/09 stalk domain generated as described previously (331) in carbonate buffer (pH=9.4). Plates were washed 3 times with PBST and blocked for at least 1h at room temperature

with 3% goat serum (Gibco) and 0.5% milk powder in PBST. Plates were washed 3 times prior to the addition of serially diluted (2-fold dilution) samples added a starting dilution of 1:100. Samples were incubated for 2h at room temperature, washed and anti-mouse IgG-peroxidase (HRP) conjugated secondary antibody was added (1:3000; A9044; Sigma) and plates were incubated for 1h at room temperature. The plates were washed 3 times and developed with *o*-phenylenediamine dihydrochloride (OPD; Sigma) and stopped after 10min using 3M HCl. Plates were read at 490nm on a plate reader. To determine the antibody titer, a cutoff value was determined by calculating the average OD values of the negative controls for each plate and adding the standard deviation. The titer for the sample was calculated as the highest serum dilution with an OD value above the cutoff.

Flow Cytometry Analysis of T cells

Lungs were excised and single-cell suspensions were generated following red blood cell lysis. Total viable cells were determined using a hemocytometer and trypan blue (Invitrogen) exclusion and resuspended at $1x10^6$ cells/ml in RPMI 1640 (Cellgro) supplemented with 10% FBS (Fisher Scientific) and 1% penicillin/streptomycin. For IAV-specific T cells enumeration, cells were cultured for 5h with IAV peptide antigen (CD8:$NP_{366-374}$, or CD4: $HA_{211-255}$, $NP_{311-325,}$ respectively) (ProImmune), PMA (Sigma) and Ionomycin (Sigma) or media alone (unstimulated) in media containing Brefeldin A (GolgiPlug[A]) The viability of cells was determined by fixable Live/Dead aqua viability dye (Invitrogen) and Fc receptors were blocked using anti-CD16/32[A]. The T cell populations were stained with the following antibodies: FITC conjugated anti-CD3 (17A2)[A], AF700 conjugated anti-CD4 (RM4-5)[A], PerCP-Cy5.5 conjugated anti-CD8 (53-6.7)[B], APC conjugated anti-CD44 (IM7)[A], eVolve 605 conjugated anti-CD62L (MEL-14)[B], eFluor 450 conjugated anti-CD69 (H1.2F3)[B], PE conjugated anti-CD103 (M290)[A], PE conjugated $D^bNP_{366-374}$ tetramer for ma2009 (ASNENVETM, NIH Tetramer Core Facility) and BV421 conjugated D^bNP366-374 tetramer for HK68 (ASNENMDAM, NIH Tetramer Core Facility). Intracellular

staining with PeCy7 conjugated anti-IFN-γ (XMG1.2)[A], FITC conjugated anti-TNF-α (MP6-XT22)[A], BV412 conjugated anti-IL4 (11B11)[A], APC conjugated IL-17 (eBio17B7)[B], was performed following permeabilization and fixation with Cytofix/Cytoperm and Perm/Wash buffer[A]. Data were acquired using a FACS Fortessa (FACS DIVA Software) and analyzed using FlowJo v.10 (Tree Star, Inc.). Total cell counts were determined by multiplying each live cell population percentage by the total live cell counts acquired prior to staining by trypan blue exclusion counts on a hemocytometer. All reagents were purchased from BD Biosciences[A] or eBioscience[B] unless stated otherwise.

Statistical Analyses

Morbidity and clinical data were analyzed with a MANOVA followed by planned comparisons. Antibody titers, virus titers, and histopathological data were analyzed using two-way ANOVAs or t-tests and significant interactions were further analyzed using the Tukey method for pairwise multiple comparisons. Survival was analyzed using a Kaplan Meyer survival curve followed by a log-rank test. Mean differences were considered statistically significant if $P<0.05$.

Figure and table legends

Figure 3.1: Progesterone (P4) and levonorgestrel (LNG) treatment reduced morbidity and antibody production during primary H1N1 IAV infection. Adult female mice were treated with placebo, progesterone (P4), or levonorgestrel (LNG) and inoculated intranasally with a low dose of ma2009 H1N1 virus. Mice were monitored daily for changes in body mass (A) and clinical disease (B) for 21 days post-inoculation (dpi). Serum was collected at 14, 21, 28, and 35 dpi and anti-ma2009 IgG titers were measured by ELISA (C) and ma2009 neutralizing antibody titers were analyzed by neutralization assay (D) (n=30/treatment/time-point). Bronchoalveolar lavage (BAL) fluid was collected at 14, 21, and 28dpi and analyzed for anti-ma2009 IgA titers (E) and neutralizing antibody titers (F) (n=8-10/treatment/time-point). The stippled line represents the antibody levels for naïve animals. Data represent means ±SEM from three independent replications and significant differences between P4 and placebo are represented by asterisks (*), and between LNG and placebo by a pound sign (#).

Figure 3.2: Neither progesterone (P4) nor levonorgestrel (LNG) treatment altered protection following challenge with a ma2009 H1N1 drift variant in females. Adult female mice were treated with placebo, progesterone (P4), or levonorgestrel (LNG) and inoculated intranasally with a low dose of ma2009 H1N1 virus. Six weeks later, mice were challenged intranasally with a high dose of a ma2009 drift variant (K166Q) H1N1 virus and euthanized at the indicated time-points (A). Mice (n=10/treatment) were monitored for changes in body mass (B) for 21 days post-challenge. Broadly neutralizing stalk antibodies were measured in serum collected at 14, 21, 28, and 35 days post primary infection using a stalk antibody ELISA with an exotic HA head and conserved ma2009 H1N1 stalk (cH6/1) (C; n=25-30/treatment/time-point). Neutralizing antibody responses (D) and IgG titers (E) against the primary ma2009 H1N1 virus and were measured in serum collected prior to (pre-challenge; 21 days post primary infection) and 21 days post-challenge (n=10/treatment/time-point). Neutralizing antibody responses (F) and

IgG titers (G) against ma2009 drift variant (dv) H1N1 virus were measured in serum collected prior to (pre-challenge; 21 days post primary infection) and 2, 6, 14, or 21 days post-challenge (n=10/treatment/time-point). The stippled line represents the antibody levels for naïve animals. Data represent means ±SEM from two independent replications and significant differences between P4-treated and placebo-treated females are represented by asterisks (*), between LNG-treated and placebo-treated females by a pound sign (#), and between pre- and post-challenge within a treatment group by a plus sign (+).

Figure 3.3: Progesterone (P4) and levonorgestrel (LNG) treatments reduced survival, but increased antibody titers following challenge with a heterosubtypic H3N2 influenza A virus. Adult female mice were treated with placebo, P4, or LNG and inoculated intranasally with a low dose of ma2009 H1N1 virus. Six weeks later, ma2009 H1N1 infected mice were challenged intranasally with a high dose of HK68 H3N2 virus and euthanized at the indicated days post-challenge (A). These female mice (n=15/treatment) were monitored for changes in mortality (B) and body mass (C) for 21 days post-challenge. Infectious virus titers in the lungs were measured at 2, 4, 6, and 8 days post-challenge (D; n=5-8/treatment/day). Neutralizing antibody titers (E) against the primary ma2009 H1N1 virus were measured in serum collected prior to (pre-challenge; 21 days post primary infection) and 21 days post-challenge (n=10/treatment/time-point). Neutralizing antibody responses (F) and IgG titers (G) against the HK68 H3N2 virus were measured in serum collected prior to (pre-challenge; 21 days post primary infection) and 6, 14, or 21 days post-challenge (n=10/treatment/time-point). The stippled line represents the antibody levels for naïve animals. Data represent means ±SEM from two independent replications and significant differences between P4 and placebo are represented by asterisks (*), and between LNG and placebo by a pound sign (#).

Figure 3.4: Progesterone (P4) and levonorgestrel (LNG) treatments increased pulmonary immunopathology following challenge with a heterosubtypic H3N2 influenza A virus. Adult female mice were treated with placebo, progesterone (P4), or levonorgestrel (LNG) and inoculated intranasally with a low dose of ma2009 H1N1 virus. Six weeks later, mice were challenged intranasally with a high dose of HK68 H3N2 virus. H&E stained lung sections collected at 6 and 8 days post-challenge were scored for inflammation as a cumulative score of perivasculitis, bronchiolitis, alveolitis, edema, and necrosis (A). Separate scores at 8 days post-challenge (dpc) for perivasculitis, bronchiolitis, alveolitis, edema, and necrosis are shown (B). Representative images of overall inflammation (2X magnification) and focused areas (10X magnification) with cellular infiltration and edema are shown (C) (n=5/treatment/day). Data represent means ±SEM from two independent replications and significant differences between P4 and placebo are represented by asterisks (*), and between LNG and placebo by a pound sign (#).

Figure 3.5: Treatment with either progesterone (P4) or levonorgestrel (LGN) reduced virus-specific CD8+ T cell in the lungs of female mice challenged with a heterosubtypic H3N2 influenza A virus. Adult female mice were treated with placebo, progesterone (P4), or levonorgestrel (LNG) and inoculated intranasally with a low dose of ma2009 H1N1 virus. Six weeks later, mice were challenged intranasally with a high dose of HK68 H3N2 virus. Lung single cell suspensions were harvested for flow cytometry analysis at 42 days post primary infection (i.e., prior to challenge; labeled day 0 post-challenge), and at days 2 and 6 days post-challenge, and stimulated *ex vivo* with a ma2009-specific antigen in presence of BFA. Total numbers of live CD8+ T cells expressing IFN-γ (A), TNF-α (B), IFN-γ and TNF-α (C), total numbers of live tetramer specific CD8+ T cells (D) were quantified. Data represent means ±SEM from two independent replications (n=8/treatment/day) and significant differences between P4 and placebo are represented by asterisks (*), and between LNG and placebo by a pound sign (#).

Figure 3.6: Treatment with progesterone (P4) or levonorgestrel (LGN) reduced virus-specific memory CD8+ T cell responses in the lungs of female mice challenged with a heterosubtypic H3N2 influenza A virus. Adult female mice were treated with placebo, progesterone (P4), or levonorgestrel (LNG) and inoculated intranasally with a low dose of ma2009 H1N1 virus. Six weeks later, mice were challenged intranasally with a high dose of HK68 H3N2 virus. Lung single cell suspensions were harvested for flow cytometry analysis at 42 days post-primary infection (i.e., prior to challenge; labeled day 0 post-challenge), and at 2 and 6 days post-challenge, and stimulated *ex vivo* with a ma2009-specific NP antigen in presence of BFA. Cells were gated on lymphocytes (SSC vs FSC) and doublets (FSCH-H vs FSC-A) and dead cells (viability dye+) were excluded. CD8+ or CD4+ T cells were determined from the live cell gate. Memory T cell (CD44+CD62L-), T central memory (TCM; CD44+CD62L-) and naïve CD8+ T cells (CD44-CD62L+) were gated on the CD8+ T cell subset. Tissue resident memory (TCM) cells were gated based on their expression of CD103 and CD69 from the CD44+CD62L- memory T cell gate (A). Total numbers of live memory CD8+ T cells (B), tissue resident memory CD8+ T cells (TRM; C), central memory CD8+ T cells (TCM; D), and memory CD8+ T cells expressing IFN-γ (E), TNF-α (F) or both (G) were quantified. Data represent means ±SEM from two independent replications (n=8/treatment/day) and significant differences between P4 and placebo are represented by asterisks (*), and between LNG and placebo by a pound sign (#).

Table 3.1: Total numbers of virus-specific CD4+ and CD8+ T cell recognizing ma2009 in the lungs of female mice following challenge. Adult female mice were treated with placebo, progesterone (P4), or levonorgestrel (LNG) and inoculated intranasally with a low dose of ma2009 H1N1 virus. Six weeks later, mice were challenged intranasally with a high dose of HK68 H3N2 virus. Lung single cell suspensions were harvested for flow cytometry analysis at 42 days post primary infection (i.e., prior to challenge; referred to as day 0 post-challenge), and at days 2 and 6 days post-challenge and stimulated *ex vivo* with ma2009-specific antigen in presence

of BFA. Total numbers of live T cells were analyzed. Data represent means ±SEM from two independent replications (n=8/treatment/day) and significant differences are between P4 and placebo represented by asterisks (*), and between LNG and placebo by a pound sign (#).

Table 3.2: Total numbers of virus-specific CD8+ T cell recognizing HK68 in the lungs of female mice following challenge. Adult female mice were treated with placebo, progesterone (P4), or levonorgestrel (LNG) and inoculated intranasally with a low dose of ma2009 H1N1 virus. Six weeks later, mice were challenged intranasally with a high dose of HK68 H3N2 virus. Lung single cell suspensions were harvested for flow cytometry analysis at 42 days post primary infection (i.e., prior to challenge; labeled as day 0 post-challenge), and at days 2 and 6 days post-challenge and stimulated *ex vivo* with HK68-specific antigen in presence of BFA. Total numbers of live T cells were analyzed. Data were analyzed with two-way ANOVA followed by Tukey tests and represent means ±SEM from two independent replications (n=8/treatment/day) and significant differences are between P4 and placebo represented by asterisks (*), and between LNG and placebo by a pound sign (#).

Figure 3.1

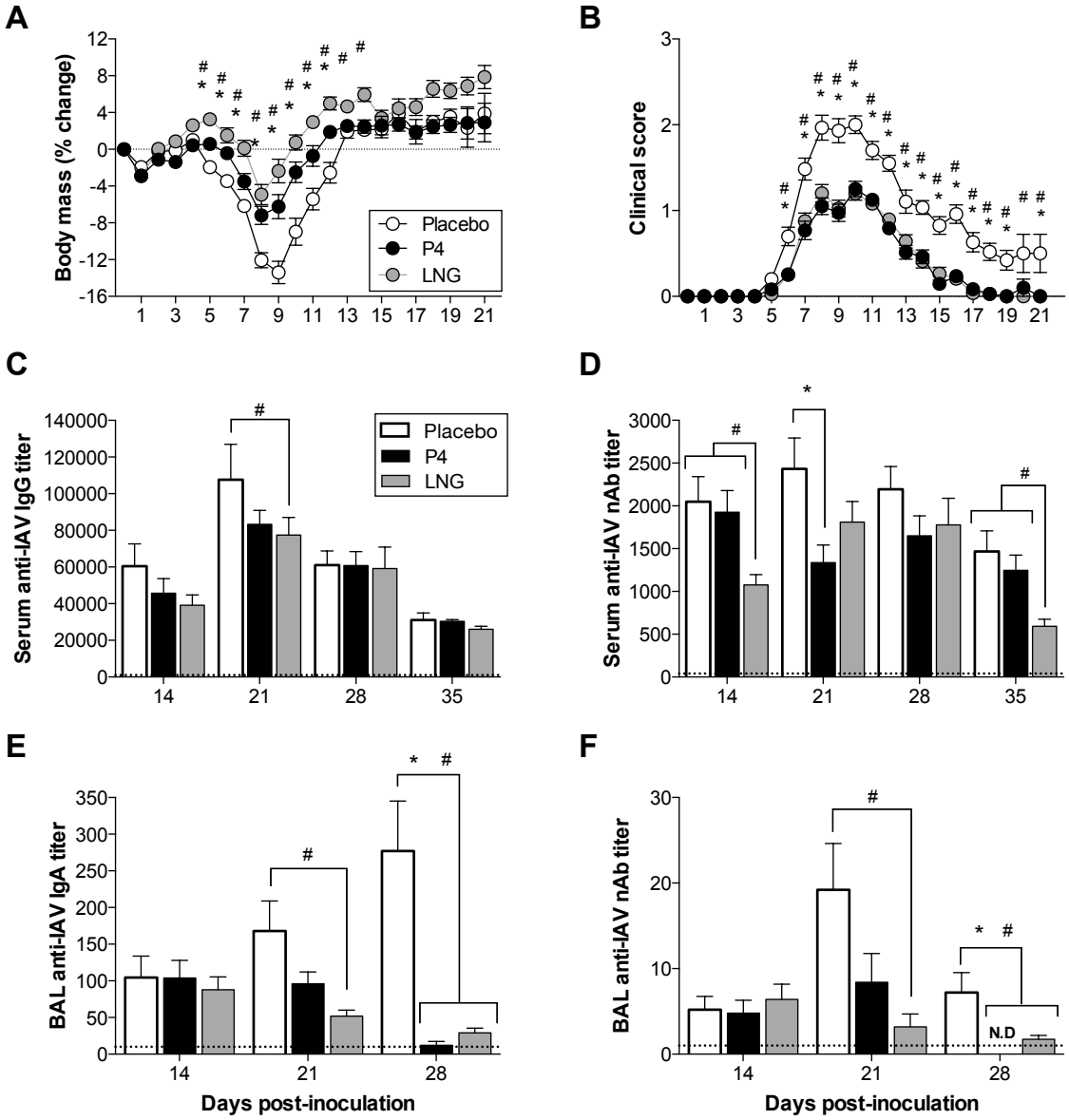
A
Body mass (% change)
Placebo
P4
LNG
B
Clinical score
C
Serum anti-IAV IgG titer
Placebo
P4
LNG
D
Serum anti-IAV nAb titer
E
BAL anti-IAV IgA titer
Days post-inoculation
F
BAL anti-IAV nAb titer
N.D
Days post-inoculation

Figure 3.2

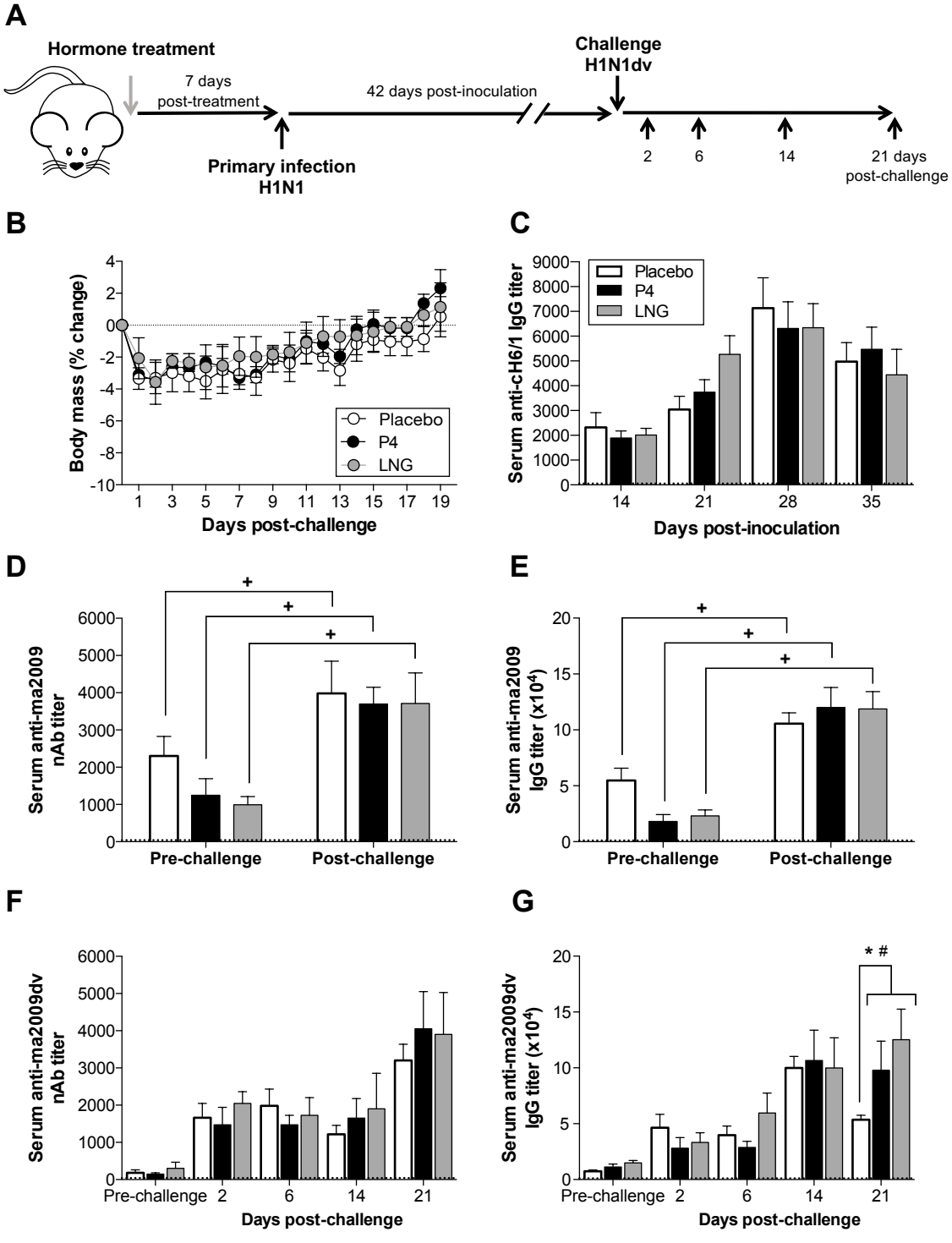
A
Hormone treatment
7 days
post-treatment
42 days post-inoculation
Challenge
H1N1dv
Primary infection
H1N1
2
6
14
21 days
post-challenge
B
Body mass (% change)
Placebo
P4
LNG
Days post-challenge
C
Serum anti-cH6/1 IgG titer
Placebo
P4
LNG
Days post-inoculation
D
Serum anti-ma2009
nAb titer
Pre-challenge
Post-challenge
E
Serum anti-ma2009
IgG titer (x10⁴)
Pre-challenge
Post-challenge
F
Serum anti-ma2009dv
nAb titer
Pre-challenge
Days post-challenge
G
Serum anti-ma2009dv
IgG titer (x10⁴)
Pre-challenge
Days post-challenge

Figure 3.3

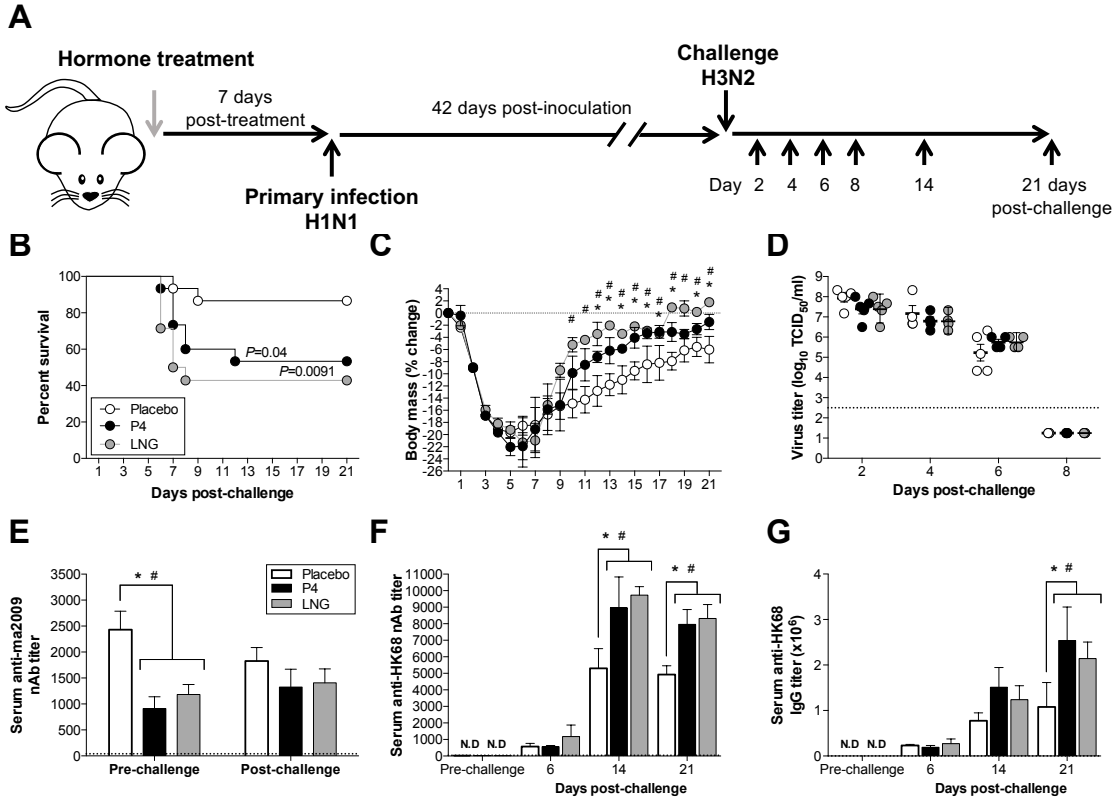
A
Hormone treatment
7 days
post-treatment
42 days post-inoculation
Challenge
H3N2
Primary infection
H1N1
Day 2 4 6 8 14
21 days
post-challenge
B
Percent survival
P=0.04
P=0.0091
Placebo
P4
LNG
Days post-challenge
C
Body mass (% change)
D
Virus titer (log10 TCID50/ml)
Days post-challenge
E
Serum anti-ma2009 nAb titer
Placebo
P4
LNG
Pre-challenge
Post-challenge
F
Serum anti-HK68 nAb titer
N.D N.D
Pre-challenge
Days post-challenge
G
Serum anti-HK68 IgG titer (x10^6)
N.D N.D
Pre-challenge
Days post-challenge

Figure 3.4

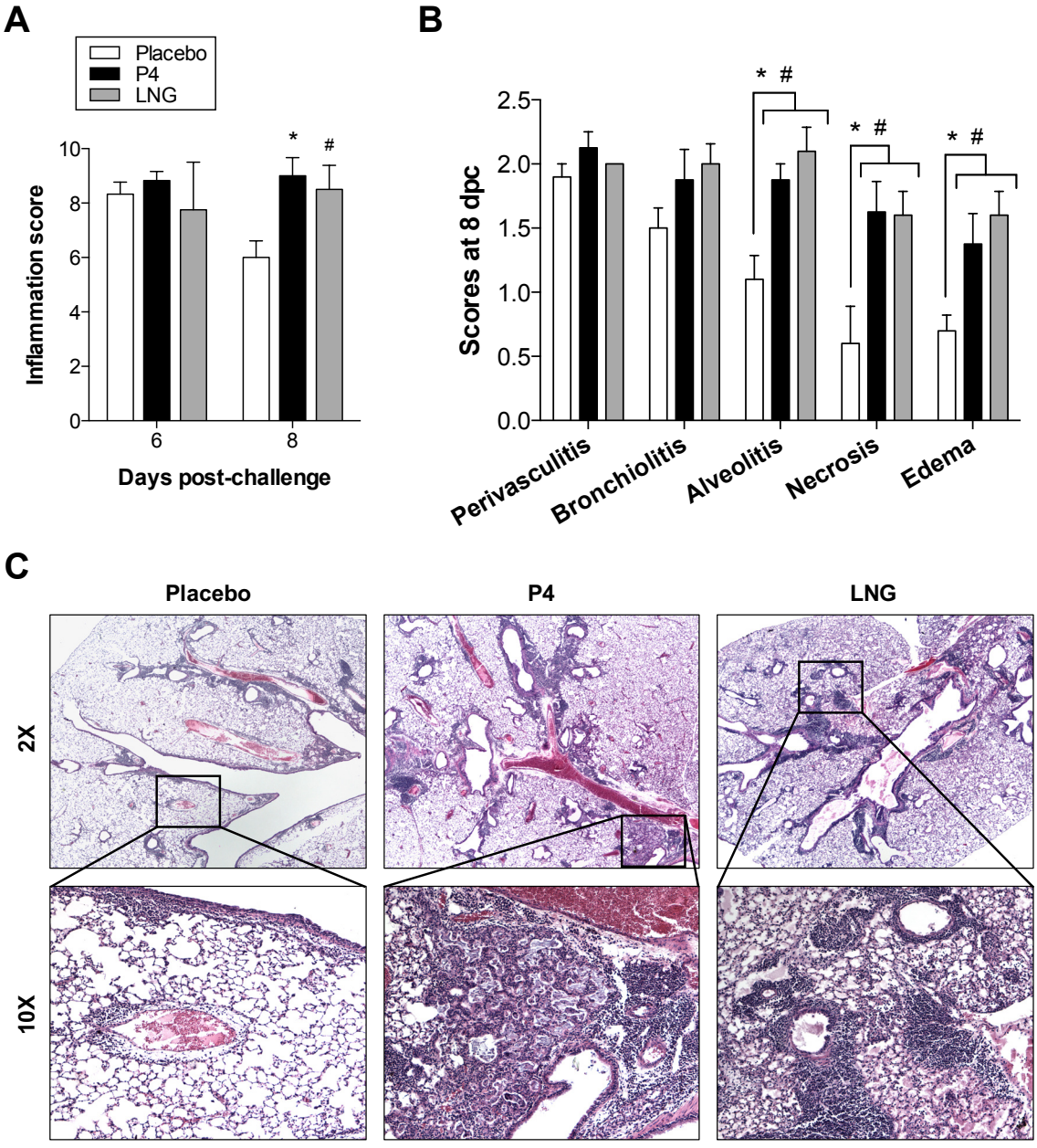
A
Placebo
P4
LNG
Inflammation score
Days post-challenge
B
Scores at 8 dpc
Perivasculitis
Bronchiolitis
Alveolitis
Necrosis
Edema
C
Placebo
P4
LNG
2X
10X

Figure 3.5

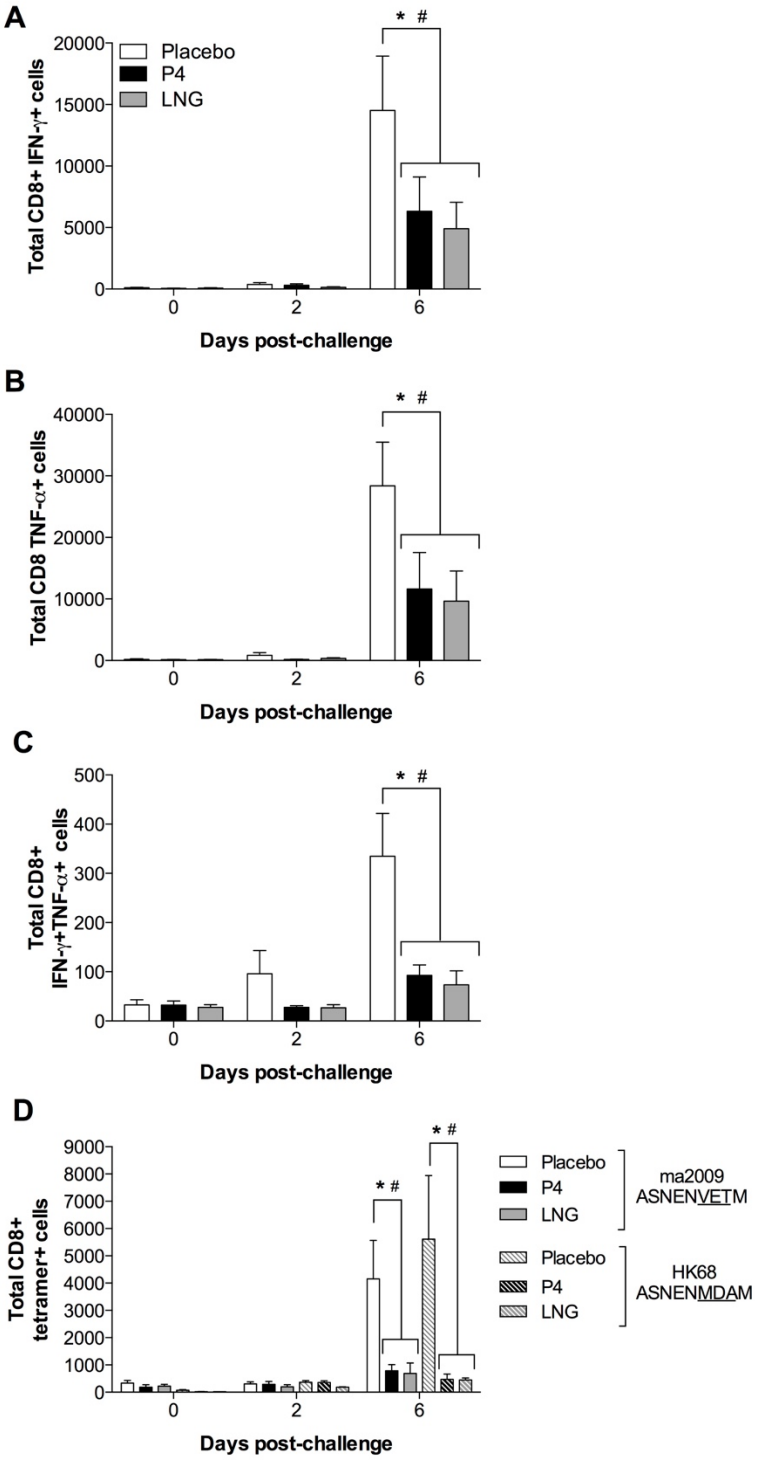
A
Placebo
P4
LNG
Total CD8+ IFN-γ+ cells
Days post-challenge
B
Total CD8 TNF-α+ cells
Days post-challenge
C
Total CD8+ IFN-γ+TNF-α+ cells
Days post-challenge
D
Total CD8+ tetramer+ cells
Days post-challenge
ma2009 ASNENVETM
HK68 ASNENMDAM

Figure 3.6

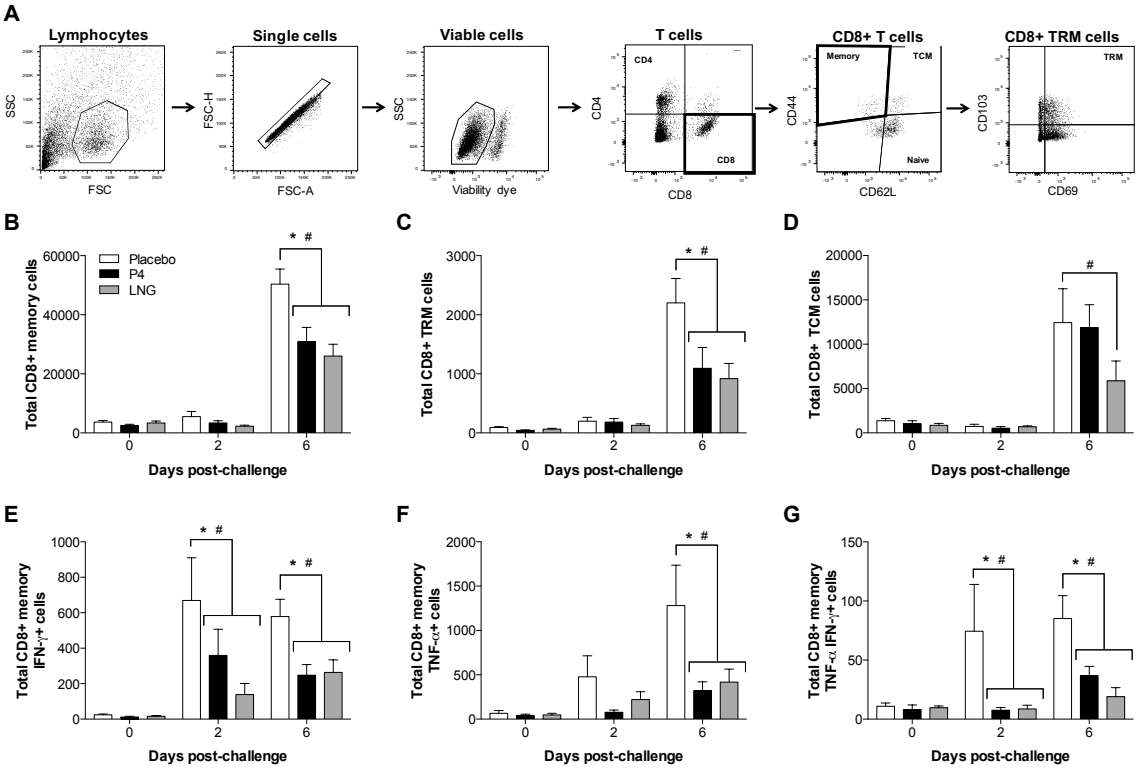
A
Lymphocytes
SSC
FSC
Single cells
FSC-H
FSC-A
Viable cells
SSC
Viability dye
T cells
CD4
CD8
CD8+ T cells
Memory
TCM
Naive
CD44
CD62L
CD8+ TRM cells
TRM
CD103
CD69
B
Placebo
P4
LNG
Total CD8+ memory cells
Days post-challenge
C
Total CD8+ TRM cells
Days post-challenge
D
Total CD8+ TCM cells
Days post-challenge
E
Total CD8+ memory IFN-γ+ cells
Days post-challenge
F
Total CD8+ memory TNF-α+ cells
Days post-challenge
G
Total CD8+ memory TNF-α IFN-γ+ cells
Days post-challenge

Table 3.1

Total cells	Treatment	Days Post-Challenge 0	2	6
CD4+ T cells	Placebo	8230±662	6829±1480	32752±3067
	P4	8093±1720	9511±1951	29904±3816
	LNG	7018±857	7017±1424	27204±3200
IFN-γ+ CD4+ T cells	Placebo	86.35±9.2	185.7±32.3	883±114
	P4	93.04±21.1	244±35.1	**551.6±101 ***
	LNG	83.63±12.2	159.8±35.3	**454±81.3 #**
IL-4+ CD4+ T cells	Placebo	19.72±5.6	8.71±4.0	22.84±6.1
	P4	28.36±10.8	14.88±7.8	26.75±5.4
	LNG	20.72±7.6	5.96±2.8	18.63±4.4
IL-17+ CD4+ T cells	Placebo	46.76±14.2	26.62±9.5	34.24±11.0
	P4	67.85±24.5	33.64±7.7	29.27±11.3
	LNG	42.76±24.2	41.68±11.3	31.31±12.3
CD8+ T cells	Placebo	8958±823	11275±2590	55003±13269
	P4	9798±400	15512±5861	44293±7757
	LNG	12192±2273	12215±1289	45214±9833
Naïve CD8+ T cells	Placebo	4413±608	3500±1271	5530±380
	P4	4372±1272	2365±650	5633±814
	LNG	2815±817	3360±283	4800±1126

Table 3.2

Total T cells	Treatment	Days Post-Challenge 0	2	6
IFN-γ+ CD8+	Placebo	73.3±17.3	246.7±92.7	12183±4127
	P4	52.5±11.6	352.9±124.9	6993±31.22
	LNG	65.3±13.1	235.7±85.6	7204±125.6
TNF-α+ CD8+	Placebo	209.4±78.6	1125±582	26323±8405
	P4	171.0±42.0	207.1±25.74	**10609±5160 ***
	LNG	120.2±39.6	1296±586.7	**12849±5691 #**
IFN-γ+TNF-α+ CD8+	Placebo	37.5±8.8	74.7±39.3	262.8±41.9
	P4	28.9±4.6	25.39±4.5	**107.0±23.6 ***
	LNG	21.7±3.8	53.56±43.4	**66.09±12.2 #**
Memory CD8+	Placebo	3462±566.2	5395±1793	47865±5875
	P4	2523±627.0	3439±765.4	**26862±5207 ***
	LNG	3384±556.5	4548±1152	**23661±4660 #**
TRM CD8+	Placebo	82.0±14.6	167.6±66.8	1843±449.3
	P4	51.7±18.2	176.0±53.6	**826.6±318.6 ***
	LNG	64.3±9.8	152.5±46.0	**710.8±258.4 #**
TCM CD8+	Placebo	1765±401.3	842.0±297.0	12804±2494
	P4	1261±277.9	617.0±206.1	**8660±2075 ***
	LNG	1404±259.6	1174±121.8	**5641±710.0 #**
IFN-γ+ CD8+ Memory	Placebo	21.0±7.1	365.1±164.7	444.3±96.3
	P4	11.9±4.6	342.5±120.3	258.8±98.8
	LNG	17.0±3.9	232.9±119.3	242.0±52.0
TNF-α+ CD8 Memory	Placebo	106.5±48.8	706.9±376.2	1071±209.1
	P4	60.1±14.7	**81.3±26.8 ***	**373.8±94.1 ***
	LNG	45.2±16.5	398.7±259.8	**320.0±84.9 #**
IFN-γ+ TNF-α+ CD8 Memory	Placebo	12.1±2.1	79.2±50.3	45.9±10.4
	P4	7.7±1.1	**11.15±3.8 ***	20.96±8.2
	LNG	7.7±2.9	**28.2±16.8 #**	16.5±5.3

Chapter 4

GENERAL DISCUSSION

Olivia J. Hall

The research within this dissertation illustrates the need for a better understanding of the role of sex steroid hormones in infectious diseases. More precisely, the findings of this dissertation demonstrate a novel mechanism for progesterone in the protection of IAV-infected mice by decreasing inflammation, promoting repair and altering memory and antibody responses. These findings may be applicable for other disease models and their implication in human clinical research remains to be further explored.

Progesterone protects female mice from IAV infection

Severe infections with IAV may lead to excessive inflammation and pulmonary damage that can cause severe disease and even death (153, 264, 265). In chapter 2, I made the unique observation that treatment of female mice with a host factor, the sex steroid hormone P4, can prevent immunopathology and decrease mortality following lethal IAV infection. This observation was expanded to female mice infected with a sub-lethal dose of IAV, and in addition to decreasing inflammation and immunopathology, P4 promotes repair and leads to faster recovery. This is consistent with the literature which demonstrates an anti-inflammatory and repair role of P4 in infectious disease and in autoimmune diseases such as multiple sclerosis (39, 44, 64, 67, 95, 101). As highlighted in the introductory chapter, the anti-inflammatory role of P4 may be a disadvantage when a strong pro-inflammatory response is needed, however, in the context of IAV infection where there is a tendency of the host to induce excessive inflammation in response to the infection, treatment with P4 and regulation of the inflammation is beneficial.

Progesterone promotes pulmonary repair following IAV infection

Mice infected with IAV suffer from extensive lung tissue damage due to the cytokine storm initiated by the host immune response to clear the virus (264, 265). This pulmonary injury needs to be rapidly repaired following virus clearance, and inflammation dampened in order for the lungs to function optimally and for full recovery of the mice. Several mechanisms of

pulmonary repair following IAV infection have been elucidated and all concern host factors that come in place during the infection to promote proliferation of epithelial cell and pulmonary tissue remodeling. In mice treated with P4, four different repair mechanisms involving host proteins promote tissue remodeling following IAV infection: upregulation of TGF-β, increase in regulatory CD39+ Th17 cells, upregulation of IL-22 and increases in amphiregulin (AREG) production.

TGF-β and repair

TGF-β has a potent role in repair and homeostasis of injured tissues and is produced by a vast variety of cell types including epithelial cells and immune cells, each of which could potential respond to P4 and induce production of TGF-β during IAV infection. I hypothesize that P4 may exert its effects by acting synergistically on multiple cell types at once to produce TGF-β. This could be assessed by looking at the total numbers of PR+ cells producing TGF-β by flow cytometry in the lungs of IAV infected mice and comparing P4 treatment to placebo. Any cell types identified to produce TGF-β in response to P4 treatment during IAV infection could then be further investigated by using PR-floxed mice to knockout the PR from each of these cell types and measure any differences in lung pathology and pulmonary repair.

The role of suppressive Th17 cells in repair

Th17 cells are generally considered pro-inflammatory and exacerbate inflammatory diseases such as inflammatory bowel disease (IBD), collagen-induced arthritis and EAE (332, 333). Recently, a population of Th17 cells has been shown to have a regulatory role similar to that of Tregs (306, 307). They are characterized by the expression of the ectonucleotidases CD39 which allows the conversion of ATP to adenosine and dampens inflammation in lungs and other tissues (306, 334-338). The expression of CD39 on these cells is dependent on the presence of TGF-β and IL-6 which we also show to be increased following P4 treatment of IAV-infected

mice (131, 306). Regulatory CD39+ Th17 cells are increased following P4 treatment of IAV-infected mice to decrease overall inflammation and induce the production of IL-22 to promote the repair of epithelial cells, but the precise role of these cells remains to be further explored. The manipulation of CD39+ Th17 cells poses technical difficulties as many other cell types also express CD39, including monocytes macrophages, DCs, and Tregs (334, 336, 339)**.** Therefore, removing all cells expressing CD39 will not allow for a better understanding of the exact role of these regulatory Th17 cells. The best way to further characterize these cells would be in an *in vitro* culture system using naïve CD4+ T cells differentiated into Th17 cells in the presence of P4 to assess their suppressive role in the presence of CD8+ OT-I T cells stimulated with the SINFELK peptide as compared to non-P4-treated cells, similarly to what was done by Chalmin et al. (306). Additionally, their precise role in repair could be further characterized by using differentiated CD39+ Th17 in a co-culture system with primary pulmonary epithelial cells infected with IAV, in the presence or absence of P4 and analyzing the role of the suppressive Th17 in dampening inflammation and promoting faster repair.

Progesterone promotes repair through the production of amphiregulin

Amphiregulin (AREG) is involved in pulmonary tissue remodeling and repair during many different aspects of lung pathology including lung injury, asthma, and infection (273, 279-286). In IAV infection, AREG protects mice by decreasing hypothermia, reducing protein leakage and promoting faster recovery (131, 279, 280). In chapter 2, I identified a role for P4 on inducing AREG production and the requirement of this factor for P4-induced pulmonary repair in IAV-infected mice, using both administration of AREG in P4-depleted mice to restore protection and depletion of AREG in P4-treated mice to show a defect in repair and protection when AREG is lacking. I also show that epithelial cells are the main producers of AREG and, *in vitro*, are capable of promoting P4-induced repair following a mechanical injury.

A new definition for recovery following IAV infection

All of these host factors lead to the proliferation of lung epithelial cells and help maintain the integrity of the epithelial barrier, allowing for a faster return to homeostatic conditions and improved pulmonary functions including gas exchange and airway elasticity. This research also highlights that despite complete clearance of the virus by 14dpi and return to homeostatic body mass and temperature by 21dpi, damage may persist in the lungs and hinder pulmonary lung function. Most IAV mouse studies use the return to baseline of either body mass or temperature as a measure of recovery, with studies even defining recovery at earlier stages (311, 340). These classical factors need to be reconsidered as this dissertation shows that despite a return to baseline for body mass and temperature, severe damage and inflammation may persist in the lungs and cause long-term defects in pulmonary function. This persistent pulmonary damage may also lead to increased opportunistic secondary bacterial infections which are a major concern following IAV infections (341-343). It would be interesting to test the role of secondary bacterial infections in our model of IAV infection in P4-treated mice. Despite the rapid repair of the damaged lungs, a dampening in immune responses promoted by P4 may hinder adequate responses to the bacterial challenge as observed in tuberculosis or chlamydia models of infection (94, 121, 130).

Expanding the role of P4 on repair beyond IAV infections

These repair factors also play a role in a variety of other types of lung injuries including infection with respiratory syncytial virus (RSV), acute lung injury and bacterial infections with the induction of similar pulmonary pathogenesis (274, 284, 344-346). The *in vitro* experiments from chapter 2 using mouse tracheal epithelial cells demonstrate that P4 can also promote the repair of mechanical injuries to the epithelium in the absence of a viral infection, which suggests that the protective role of P4 can be expanded beyond influenza infection and beyond viral infections all together. Studies to further understand the role of P4 in pulmonary repair are required and would involve testing P4 treatment in a non-pathogenic lung injury model such as

the bleomycin-induced lung injury model or using a model of pulmonary bacterial infection. I hypothesize that P4 would also promote the induction of similar host factors including AREG production to induce the proliferation and repair of the injured epithelium and foster a faster return to a healthy pulmonary environment. In terms of translating these findings into the clinic, administration of P4 is currently under phase 2 and phase 3 clinical trials for the treatment of traumatic brain injury and under review for clinical trial in the treatment of ischemic stroke in both men and women (29, 31, 32, 75, 347).

The benefits of the use of P4 or a synthetic progestins in the treatment of IAV infection in humans, is that unlike antivirals, it may not be necessary to administer treatment at early stage of infections which are harder to capture due to the nature of the infection. If P4 could help in the treatment of IAV infection, due to its role during the recovery phase, its value may be in cases with severe ARDS complications. The role of P4 in dampening IAV-induced infection is also non-negligible and could help prevent the development of ARDS. This needs to be initially tested in mice by administering P4 at later time-points during IAV infection, such as 7 or 14 dpi and evaluating the effects of P4 during these later stages. Additionally, further understanding of the cells expressing PR and responding to P4 is also necessary.

Characterizing the effects of P4 signaling on different cell types

A large variety of cells including NK cells, macrophages, dendritic cells (DCs), T cells as well as non-immune cells, such as epithelial cells, endothelial cells, and neuronal cells express PRs (12, 13)**.** In chapter 2, I have demonstrated that IAV-infected lungs of mice treated with P4 have increased expression of PR, that tracheal epithelial cells express PR, and that epithelial cells are the main producers of AREG and may be doing so in response to P4 signaling. I hypothesize that epithelial cells may be the main cell type responding to P4 treatment to produce repair factors such as AREG and TGF-β but this remains to be further assessed. To do so, flow cytometry

analysis of the different cell types present in the lungs following IAV infection and P4 treatment is necessary to characterize their PR expression. Furthermore, to assess whether the presence of PRs on these cells has any functional relevance, it is necessary to narrow down which cells respond to P4 and are increased as compared to placebo-treatment. A way to evaluate this involves the use of PR-floxed mice and mice expressing Cre recombinase in different cell types. However, this approach involves a great number of different combinations of cre-inducible cells and corresponding mice and would prove very timely to investigate. A better approach perhaps would be with a bone marrow chimera model using irradiated PRKO mice and performing an adoptive transfer of bone marrow hematopoietic cells from wild type mice, treating these mice with P4 and infecting them with a sub-lethal dose of IAV. If the protective effects of P4 remain, this would indicate that the presence of PR on non-hematopoietic cells such as epithelial cells is not necessary for P4 to induce its protective effects. Conversely, irradiated wild-type mice with intact PR on non-hematopoietic cells can receive a bone marrow transfer from PRKO mice to replenish the hematopoietic cell population, be treated with P4 and infected with IAV. If the protective effects of P4 remain, this would indicate that the presence of PR on non-hematopoietic stem cell is necessary and that these cells respond to P4 and mediate the protective effects. My data shows that this might be the most likely scenario as epithelial cells are the main producers of AREG.

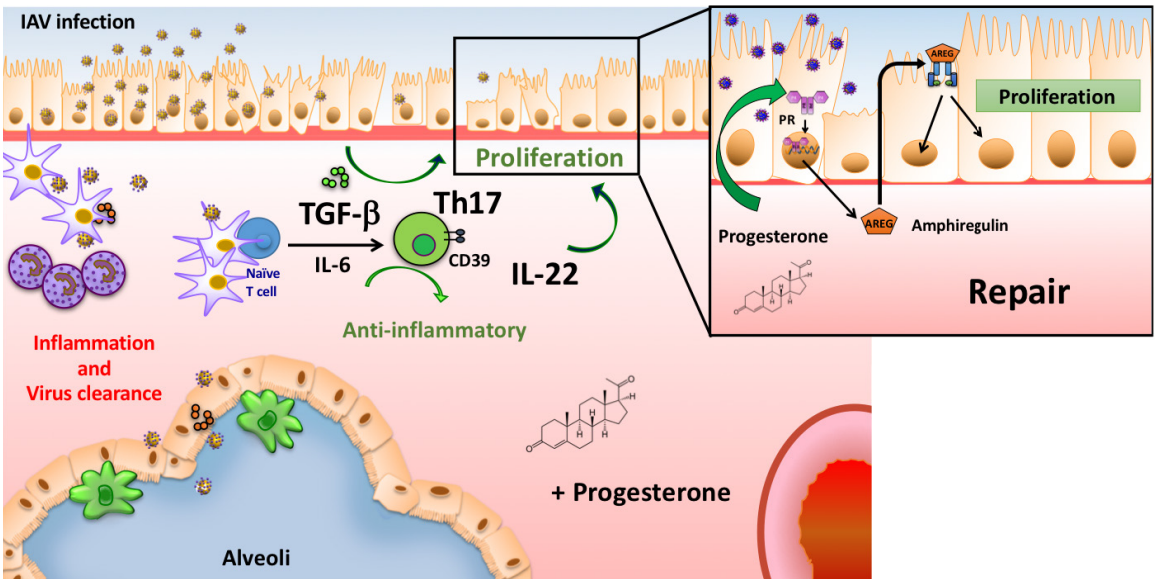

Figure 4.1: Summary of the effects of P4 on IAV pathogenesis. IAV infects airway epithelial cell which can produce pro-inflammatory cytokines and activate immune cells such as alveolar macrophages (green cells), DCs and neutrophils (in purple) that causes inflammation and along with CD8+ T cells (not depicted), lead to virus clearance. The presence of P4 leads to the production of IL-6 and TGF-β which can promote regulatory CD39+ Th17 cells differentiation and induce an anti-inflammatory milieu. Th7 cells can also produce IL-22, which, like TGF-β promote proliferation of epithelial cells and repair of the damaged epithelium. AREG is another factor that is induced by P4 signaling through the progesterone receptor (PR). AREG signals through the epidermal growth factor receptor (EGFR) to induce the proliferation of epithelial cells, which leads to repair of the damaged epithelium.

Progestins alter antibody responses

Female mice treated with either P4 or LNG experience lower titers of total anti-IAV IgG, IgA and virus neutralizing antibody titers in the serum and bronchoalveolar lavage (BAL) fluid following a primary IAV infection as compared to placebo-treated mice. These effects are even more pronounced in the BAL fluid, where levels of IgA and neutralizing antibodies are barely

detectable at 28dpi. This difference may be due to progestins hindering the ability of B cells to class-switch, or due to a difference in the numbers of antibody-secreting cells (ASCs). Very little is known on the role of progestins on B cells and this is an area that requires further investigation. To do so, total numbers of IgA+ or IgG+ B cells as well as virus-specific IgG or IgA ASCs could be evaluated by flow cytometry and ELISPOT assay respectively. This would indicate whether progestins have a direct effect on the numbers of B cells and their ability to produce either IgG or IgA. Furthermore, class-switching could also be evaluated by looking at the different subclasses of IgG, including IgG1 and IgG2c, and ratios of IgM to IgG to assess whether progestins could impact the ability of B cells to class-switch to isotypes which induce better protection from IAV (187-190).

Progestins impair memory CD8+ T cell responses following IAV infection

Following challenge with a heterosubtypic H3N2 virus, progestin-treated mice suffer from greater mortality compared to placebo-treated mice which could not be explained by differences in virus replication. Lung immunopathology in the progestin-treated mice is increased during the window in which the mice succumb to the challenge. These differences in the survival of progestin-treated mice correspond to a decrease in various populations of memory CD8+ T cells including cytokine-producing cells. Tissue-resident memory CD8+ T cells in particular, are decreased following either P4 or LNG treatment, and these cells are crucial in mediating protection against a heterosubtypic IAV challenge (206-208). Interestingly, similar studies show that control of infection in mice vaccinated and challenged with HSV-2 also require TRM (348). Progestins have been shown to decrease protection in HSV-2 challenge studies, however in these studies, TRM memory cells have not been measured (113, 118, 119). To date, no studies have evaluated the effects of progestins on the generation of memory CD8+ T cells and I hypothesize that progestin may not only affect total numbers of memory CD8+ T cells but also their phenotype. I hypothesize that less inflammation during the primary infection may both decrease

the amount of chemokines and recruitment of cells into the lungs which could then become resident memory cells along with a faster resolution of the infection which may decrease the availability of antigen and decrease antigen presentation and CD8+ T cell activation and differentiation. Additionally, the presence of progestins during the primary IAV infection may alter the interactions between naïve CD8+ T cells and antigen presenting cells. For example, progesterone decreases the expression of the co-stimulatory molecules CD80 and CD86 on DCs (33, 39) which may impact the generation of memory CD8+ T cells during a primary sub-lethal IAV infection. We observed an increase in IFN-γ- and or TNF-α-producing memory T cells but have not evaluated their cytotoxic activity which can be done by looking at their production of perforin or granzyme B by flow cytometry. Because there is no difference in pulmonary virus titers or virus clearance between the placebo- and progestin-treated mice, I hypothesize that the cytolytic activity of these memory CD8+ T cells may not be decreased following progestin treatment. Rather the quality of the response may be decreased and lack specificity in the progestin-treated mice which leads to greater overall inflammation that we observe in the form of increased pulmonary immunopathology.

Because of such differences in memory CD8+ T cell responses, it would be interesting to evaluate the longevity of these anti-influenza immune responses, as humans are thought to be re-exposed to IAVs every 5-10 years (318). With time, cellular memory responses have a tendency to wane and this could lead to even greater susceptibility following sequential IAV challenge in progestin-treated mice. As such, it would be of interest to evaluate cellular and humoral response to an IAV challenge, not six weeks post primary infection, but several months later. Because TRM confer long-lasting immunity, I hypothesize that progestin treatment may decrease the longevity of the immune response to a heterologous IAV challenge as compared to placebo treatment (349, 350). Additionally, evaluating the effects of memory CD8+ T cells in not only a

secondary infection but tertiary infection may further shed a light on the role of progestins in long-term responses to IAV.

Progestins do not affect CD4+ T cells in a heterologous IAV challenge

Mice treated with progestins show no differences in total numbers of CD4+ T cells, and no differences in numbers of Th1, Th2 and Th17 cells following heterologous IAV challenge as compared to placebo. However, the role of Tregs during a heterologous IAV challenge has not yet been evaluated. Data show that memory Tregs regulate the CD8+ T cell response, and elimination of Tregs at the time of secondary IAV challenge enhances morbidity in mice (195). It is possible that fewer Tregs are generated during the primary IAV infection in progestin-treated mice due to progestins decreasing pulmonary inflammation. These lower numbers of Tregs may then contribute to the increased inflammation observed in the progestin-treated group during the heterologous IAV challenge. In a lethal model of IAV infection there are no differences in Tregs numbers or percentages between P4- and placebo-treated mice at 7dpi. However, it is possible that the intensity of the immune response in this lethal IAV model may mask any potential differences and that evaluating Tregs at later stages and following a sublethal infection may reveal potentially differences. Additionally, CD4+ T cells are important for generating TRM (206) and therefore evaluating the numbers of CD4+ T cells following the primary IAV infection may explain why progestin-treated mice have lower numbers of CD8+ TRM.

The impact of progestins during IAV vaccination

The studies from chapter 3, explore the role of progestins in memory responses to a live IAV virus which mimics a natural infection. However, IAV vaccination is widespread and occurs annually, and therefore evaluating the role of progestins in such a model would be of great value. To do so, the memory responses, both antibodies and memory T cells, as well as responses following a lethal IAV challenge would need to be evaluated in a vaccine model with mice

infected with an inactivated IAV and treated with progestins or placebo. I hypothesize that progestins would have a similar impact following vaccination, as the immune responses involved are similar to those against a live virus despite lower levels of inflammation following infection with an inactivated virus (351). Inactivated viruses may generate lower response than a natural infection and it would be very interesting to evaluate whether the antibody responses against an inactivated virus vaccine would remain high enough to protect against a challenge with a lethal live IAV.

The impact of hormonal contraceptives on infectious diseases

As described in the introductory chapter, the role of progestins has mainly been evaluated in STIs infections and despite the widespread use of hormonal contraceptive very little research has been pursued to evaluate the role of hormonal contraceptives in viral diseases outside of the reproductive tract. Each viral infection has its characteristics but some of the cellular and molecular mechanisms are shared between viruses and the observations made in this dissertation for IAV have the potential to be expanded into other viral disease models. Very few of the studies examining the impact of progestins on infectious disease have focused on memory responses or responses to vaccination, and this is an area in particular that needs to be further expanded. This dissertation has evaluated the effects of progestins during IAV infection in a murine model, and this work needs to be translated into clinical research. Many epidemiological studies exist examining women on hormonal contraceptives and their risk of HIV acquisition or the effects of progestins on HIV disease progression (103-106). Human studies analyzing antibody responses to IAV need not only to acknowledge the sex of the subjects but their hormonal status should also be considered. Additional work in primary human pulmonary epithelial cells could also provide further insight on the role of progestins on these cells and their ability to promote repair.

Conclusion

The overall impact of this dissertation resides in its novelty. Despite the growing use of hormones since the 1960s, very little work has been conducted on their role outside of the reproductive tract, and no studies prior to these have evaluated the impact of progestins on IAV infection, pathogenesis and memory responses. With this research, I hope to raise awareness to the potential impact of progestins on respiratory infections such as influenza which could possibly be expanded to any other respiratory infection or lung injury causing damage to the lungs. Along with their anti-inflammatory and reparative role, the impact of progestins on antibody and memory responses deserves to be further explored. With over 100 million women on hormonal contraceptives and worldwide annual influenza vaccinations, knowing the impact of these progestins on antibody production is essential.

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RESEARCH EXPERIENCE

07/2012-current **Ph.D. candidate** at the Johns Hopkins Bloomberg School of Public Health (JHSPH) in the MMI department in the laboratory of Dr. **Sabra Klein**. Ph.D. Thesis studying the molecular mechanisms mediating the host immune response by progesterone during influenza infection in female mice.

02/2010-03/2011 **Master's thesis** at the Centre Universitaire Hospitalier Vaudois (CHUV) in the division of Allergy and Immunology in the laboratory of Dr. **Giuseppe Pantaleo** under the supervision of Dr. Matthieu Perreau. Master's thesis studying how Crohn's disease treatments influence Th17 cell frequencies.

Autumn 2009 **Pre-Master's research** in the Department of Biochemistry (UNIL) – OMS Centre of Research and Formation in the laboratory of Dr. **Fabienne Tacchini-Cottier**. Pre-Master's research on the role of IL-9 during infection with the parasite *Leishmania major*. Development of PCR protocols and isolation of tissue samples.

Summer 2009 **Summer undergraduate internship** at the University of Neuchâtel, Animal Physiology Department in the laboratory of Dr. **Patrick Guerin**. Study of repulsive products against ticks. *In vivo* and *in vitro* studies. Data collection and analysis.

EDUCATION

2011- current Johns Hopkins University, Bloomberg School of Public Health (Maryland, USA): **Ph.D. candidate in Molecular Microbiology and Immunology**.

2009 – 2011 University of Lausanne, School of Biology and Medicine (Switzerland): **Master of Science (MSc) in Medical Biology** - Immunology and Cancer.

2006 – 2009 University of Neuchâtel, Faculty of Science (Switzerland): **Bachelor of Science (BSc) in Biology.**

PUBLICATIONS

1. **Hall OJ**, Limjunyawong N, Vermillion MS, Robinson DP, Wohlgemuth N, Pekosz A, Mitzner W, and Klein SL. Progesterone-based therapy protects against influenza by

promoting repair in respiratory tissues and inducing recovery in females. *PLoS Pathog* 2016 Sept 12(9): e1005840

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2. vom Steeg LG, Vermillion MS, **Hall OJ**, Alam O, McFarland R, Chen H, Zirkin B and Klein SL. Age and testosterone mediate influenza pathogenesis in male mice. Accepted at the *American Journal of Physiology Lung Cellular and Molecular Physiology*.
3. Han L., Limjunyawong N., Li Z., **Hall OJ,** Mitzner W, Undem BJ, Canning BJ, Dong X. An Itch Receptor Contributes to Asthma Symptoms. Submitted to *Nature Neuroscience,* in review.
4. **Hall OJ**, Nachbagauer R., Vermillion MS, Fink AL, Phuong V, Hirsh A, Krammer F and Klein SL. Progesterone-based contraceptives suppress adaptive immune responses and decreased original antigenic sin responses to sequential infection with influenza A viruses in female mice. Submitted to *Journal of Virolog.* In review
5. **Hall OJ,** Klein SL. Progestins alter immune responses and susceptibility to infections at diverse mucosal sites. *Mucosal Immunology*. In preparation
6. **Hall OJ,** Fink AL, Vermillion MS, vom Steeg LG, Pekosz A, and Klein SL. Evaluating the effects of sex and steroid hormones on the outcome of viral infections using animal and cell culture systems. Invited manuscript for the *Journal of Visual Experiments*. In preparation.
7. **Hall OJ,** Kuok DIT**,** Vermillion MS, vom Steeg LG, Fink AL, Chan MCW, and Klein SL. Sexual dimorphism in the effects of amphiregulin-induced pulmonary repair during influenza A virus infection. *Biology of Sex Differences.* In preparation.
8. Robinson DP, **Hall OJ**, Nilles TL, Bream JH and Klein SL. 17beta-Estradiol Protects Females against Influenza by Recruiting Neutrophils and Increasing Virus-Specific CD8 T Cell Responses in the Lungs. *J Virol*. 2014 May;88(9):4711-20.
9. Ntranos A, **Hall O**, Robinson DP, Grishkan IV, Schott JT, Tosi DM, Klein SL, Calabresi PA, Gocke AR. FTY Impairs CD8 T-cell Function Independently of the Sphingosine-1-phosphate Pathway. *J Neuroimmunol.* 2014 May;270(1-2):13-21.
10. Perreau M, Welles HC, Harari A, **Hall O**, Martin R, Maillard M, Dorta G, Bart PA, Kremer EJ, Tartaglia J, Wagner R, Esteban M, Levy Y, Pantaleo G. DNA/NYVAC vaccine regimen induces HIV-specific CD4 and CD8 T-cell response in intestinal mucosa. *J Virol.* 2011 Oct;85(19):9854-62.

BOOK CHAPTER

Peretz J, **Hall OJ**, Klein SL. (2015) Sex Differences in Influenza Virus Infection, Vaccination, and Therapies. Klein SL and Roberts CW, eds. *Sex and gender differences in infection and treatments for infectious diseases*. Springer Verlag.

PRESENTATIONS

1. **Hall OJ,** Limjunyawong N, Vermillion MS, Robinson DP, Wohlgemuth N, Pekosz A, Mitzner W, and Klein SL. Progesterone protects against influenza by promoting repair and recovery in female mice. Invited Oral Presentation at the Molecular and Integrative

Physiological Science department seminar at the Harvard T.H. Chan School of Public Health, Boston, MA, November 2016.

2. **Hall OJ** and Klein SL. Progesterone-based contraceptives alter immune responses and cross-protection against influenza A viruses in female mice. Poster presentation at the 2016 Centers of Excellence for Influenza Research and Surveillance (CEIRS) Annual Meeting, Memphis, TN, July 2015
3. **Hall OJ**, Limjunyawong N, Vermillion MS, Robinson DP, Mitzner W, and Klein SL. Progesterone Protects Against Influenza by Upregulating Amphiregulin and Promoting Lung Repair and Recovery in Females. Oral presentation at the 2016 American Society of Virology (ASV) meeting, Blacksburg, VA, June 2016.
4. **Hall OJ** and Klein SL. Progesterone-based Contraceptives Alter Immune Responses and Cross-Protection Against Influenza A Viruses in Female Mice. Poster Presentation at the 2016 Johns Hopkins Vaccine Day, Baltimore, MD, March 2016. Second place at poster competition.
5. **Hall OJ,** Limjunyawong N, Vermillion MS, Mitzner W, and Klein SL. The Effects of Progesterone on Pulmonary Repair During H1N1 Infection. Poster Presentation at the 2015 Centers of Excellence for Influenza Research and Surveillance (CEIRS) Annual Meeting, Rochester, NY, July 2015
6. Vom Steeg LG, **Hall OJ**, Alam O, McFarland R, Zirkin B & Klein SL. Testosterone Regulates the Outcome of Influenza by Altering Adaptive Immune Responses in Male Mice. Poster presentation at the annual meeting of the American Society for Virology, Western Ontario, Canada, July 2015.
7. **Hall OJ,** Robinson DP, Klein SL. Progesterone Dampens Inflammation during Influenza A Virus Infection in Female Mice. Poster presentation at the 2015 Keystone Symposia on Viral Immunity, Breckenridge, CO, January 2015.
8. Glenn J, Smith M, **Hall OJ**, Klein SL, Whartenby K. CNS Autoimmunity Predisposes to Increased Influenza Morbidity. Poster presentation at the 2015 Keystone Symposia on Viral Immunity, Breckenridge, CO, January 2015.
9. **Hall OJ,** Robinson DP, Klein SL. What Your Mother Never Told You About the Pill. Invited oral Presentation at the annual Molecular Microbiology and Immunology retreat, Baltimore, MD, September 2014.
10. **Hall OJ,** Robinson DP, Klein SL. Progesterone Protects Female Mice Against Influenza A Virus Infection. Oral presentation at the 2014 American Society of Virology (ASV) meeting, Fort Collins, CO, June 2014
11. **Hall OJ,** Robinson DP, Klein SL. Progesterone Protects Female Mice Against Influenza A Virus Infection. Oral Presentation at the John's Hopkins Women's Health Research Group's (WHRG) Symposium, Baltimore, MD, May 2014.
12. **Hall OJ,** Robinson DP, Klein SL. Progesterone Protects Female Mice Against Influenza A Virus Infection. Poster presentation at the 2014 Delta Omega Poster Competition, JHSPH, Baltimore, MD, February 2014
13. Ntranos A, Grishkan IV, Robinson DP, **Hall OJ**, Klein SL, Calabresi, PA, Gocke, AR. Fingolimod Inhibits Cytotoxic T Cells: A Novel Immunomodulatory Effect of the Unphosphorylated Compound. Oral presentation at the 2013 Americas Committee for Treatment and Research in Multiple Sclerosis (ACTRIMS) meeting, Orlando, FL, June 2013.

14. Ntranos A, Grishkan IV, Robinson DP, **Hall OJ**, Klein SL, Calabresi, PA, Gocke, AR. Dissecting fingolimod's action: a novel immunomodulatory effect of the unphosphorylated compound on cytotoxic T cell function. Oral presentation at the 2013 Annual American Association of Immunologists (AAI) meeting, Honolulu, HI, May 2013.
15. **Hall OJ**, Robinson DP, Klein SL. The Pregnancy-Associated Hormones, Estriol and Progesterone, Have Differential Effects on the Outcome of Influenza A Virus Infection in C57BL/6 Female Mice. Oral presentation at the John's Hopkins Women's Health Research Group's (WHRG) Symposium, Baltimore, MD, May 2013.
16. **Hall OJ**, Robinson DP, Klein SL. The Pregnancy-Associated Hormones, Estriol and Progesterone, Have Differential Effects on the Outcome of Influenza A Virus Infection in C57BL/6 Female Mice. Poster presentation at the 2013 Organisation for the Study of Sex Differences (OSSD) meeting, Weehawken, NJ, April 2013.

HONORS AND AWARDS

- **American Society for Virology (ASV) Student Travel Grant**. Received for an oral presentation at the 2016 ASV annual meeting, April 2016.
- **Centers for Excellence in Influenza Research and Surveillance (CEIRS) Training Award.** Received for training program at the Icahn School of Medicine Mount Sinai, NY with Dr. Florian Krammer, February 2016.
- **Johns Hopkins School of Public Health (JHSPH) Student Conference Fund Award.** Received for an Oral Presentation at the 2014 ASV annual meeting, CO, June 2014.
- **Oral Abstract Award** received for an oral presentation at the Johns Hopkins Women's Health Research Group's Symposium, Baltimore, MD, May 2014.
- **The** Keerti **V. Shah Fund Honorary Scholarship**. Received for 2013-2014 at the Johns Hopkins School of Public Health in May 2013.
- **Oral Abstract Award** received for an oral presentation at the Johns Hopkins Women's Health Research Group's Symposium, Baltimore, MD, May 2013.
- **The Carlton and Estelle Herman Award in Parasitology, Vector Biology and Animal-Borne Diseases**. Received for 2012-2013 at the Johns Hopkins School of Public Health in May 2012.

AD HOC JOURNAL REVIEW

Journal of Virology, *FASEB*, and *PLoS One*

PROFESSIONAL MEMBERSHIPS

- American Society of Microbiology (**ASM**): 2012-current
- Organization for the Study of Sex Differences (**OSSD**): 2013-current
- American Society of Virology (**ASV**): 2014-current
- American Association of University Women (**AAUW**): 2012-2015
- Association for Women in Science (**AWIS**): 2014-current

TEACHING EXPERIENCE

Teaching assistant: Exam correction, review sessions and personal meeting with students for graduate-level courses by Dr. Alan Scott at the Johns Hopkins Bloomberg School of Public Health.

- Principals of Immunology I and II, 2014 and 2015
- Topics in Immunology I and II, 2014 and 2015
- Immunology, Infection and Disease, 2014 and 2015

Mentoring and training:

- Ross McFarland, Ph.D. rotation student, MMI (JHSPH), Term 3 2013
- Rebecca Yee, Ph.D. rotation student, MMI (JHSPH), Term 1 2013
- Landon vom Steeg, Ph.D. rotation student, MMI (JHSPH), Term 2/3 2013-14
- Ornob Alam, Master student, MMI (JHSPH), 2013-2015. The Effects of Elevated Testosterone on the Outcome of ma2009 H1N1 Influenza A Virus infection in Old Male Mice
- Phil Salvatore, Ph.D. rotation student, MMI (JHSPH), Term 4 2015
- Caroline Mejia-De Jesus, Summer Fellow in the James A. Ferguson Emerging Infectious Disease Fellowship program, Summer 2015
- Jamaiha Thomas, summer intern at the Johns Hopkins Summer Jobs Program, Summer 2015 and 2016
- Kyla Britson, Ph.D. rotation student, Cellular and Molecular Medicine (JHMI), Term 1 2015
- Angela Chen, Ph.D. rotation student, MMI (JHSPH), Term 3 2016
- Vanessa Phuong, summer student (undergraduate), Johns Hopkins University, Summer 2016.

RESEARCH METHODS AND SCIENTIFIC TRAINING

Languages **French**: Native language
English: Native language
German: Fluent (speaking, reading, writing. 9 years of study)

Cell culture: Cell harvesting from tissues and blood isolated from mice and humans, isolation of specific cell populations, cell line cultures, *in vitro* lymphocyte stimulation with pathogens, APC co-culture, *in vitro* cell differentiation, and virus titration ($TCID_{50}$). Isolation and differentiation of mouse tracheal epithelial cells.

Cell biology: Fluorescence-activated cell sorting (FACS, Calibur and LSRII), extra/intracellular cytokine staining, FLOWJO analysis, proliferation assays (^{3}H-thymidine and CFSE), ELISA, ELISPOT, Luminex assays, transfection, virus neutralisation assay, Immunohistochemistry and immunofluorescence microscopy. Assay development and troubleshooting.

Molecular biology: RNA extraction, cDNA synthesis, RT-PCR, Influenza, HIV and adenovirus manipulation, virus purification and culture, magnetic-activated cell sorting.

Protein analysis: 2D-PAGE, HPLC, peptide synthesis (FMOC), and Mass spectrometry.

Animal work: Rodent handling (rats and mice), survival surgery in mice (gonadectomy), subcutaneous implantation of hormone pellets, body weight and temperature monitoring, injections, intranasal infection, dissection, lung fixation, blood collection, behavioural assessments and knock-out breeding techniques.

Clinical research: Knowledge of ethics, study designing, and basic nursing skills (blood collection, injections and phlebotomy).

Computer skills: Excel, PowerPoint, Word, Windows, Mac, FlowJo, GraphPad Prism, Systat, SigmaPlot, Adobe Illustrator.

STUDENT ORGANIZATIONS

JHSPH Student Assembly – **Member at large**: 2015-2016

JHSPH Student Assembly – **Department Representative**: 2014-2015

MMI Department (JHSPH) – **Student Group Member**: 2011-2016